REMARKS

Claims 1-21 had been pending and claims 8 and 9 were examined. Claims 1-7 and 10-21 have been cancelled without prejudice or disclaimer. Applicants reserve the right to pursue the cancelled subject matter in future applications. Claims 8 and 9 have been amended, and claims 22-24 have been added. Thus, per this amendment, claims 8-9 and 22-24 are pending.

Claims 8 and 9 have been amended to recite "screening for a test compound or a salt thereof that changes a binding property of lysophosphatidic acid or a salt thereof to an EDG-2 receptor or a salt thereof; wherein the EDG-2 receptor comprises the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence with at least 95% homology to the amino acid sequence of SEQ ID NO:1; comprising the steps of: a) bringing into contact the lysophosphatidic acid, the EDG-2 receptor, and the test compound; b) measuring the binding property of the lysophosphatidic acid and the EDG-2 receptor; c) determining whether the test compound changes the binding property of the lysophosphatidic acid and the EDG-2 receptor; and d) determining whether the test compound inhibits mesangial cell growth." Support for amendments to claims 8 and 9 can be found in the specification, for example, at least at page 8, line 29 to page 9, line 25; page 32, line 21 to page 35, line 8; and page 37, line 8 to page 38, line 8. Thus, the amendments to claims 8 and 9 are fully supported by the specification.

New claims 22 and 23 depend from claims 8 and 9, respectively. Support for claims 22 and 23 can be found in the specification, for example, at least at page 8, line 18 to page 9, line 35. New claim 24 depends from claim 8. Support for claim 24 can be found in original claim 8, and in the specification, for example, at least at page 3, line 35 to page 4, line 7; page 33, line 32 to page 34, line 9; and page 59, line 35 to page 60, line 11; and at Figure 1. Thus, the new claims are fully supported by the specification.

Applicants address below each issue raised in the Office Action of July 9, 2007.

Preliminary Matters

Applicants thank the Office for considering the IDS submitted June 6, 2005.

Claim Rejections

I. Rejection of claims 8 and 9 under 35 U.S.C. § 112, First Paragraph -- Enablement

Claims 8 and 9 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. The Office alleged that "[t]he claims contain[] subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention." Action at page 3. Relying on the *Wands* factors, the Office stated that "[d]ue to the unpredictability of the art with regard to the use of LPA/EDG-2 binding modulators, the lack of guidance and working examples from the inventor, the quantity of the experimentation necessitated to establish the feasibility of the modulator to be used in treatment of kidney diseases would be considered undue." *Id.* at page 4.

Specifically, the Office stated that "[i]n order for the potential modulator to be used as claimed there has to exist a level of knowledge as the role played by the EDG-2 receptor in kidney diseases." *Id.* at page 4. The Office contended that "the correlation between the presence of the receptor in the kidney, and the diseases mentioned in the claim was not known and to this date is not known if there is any." *Id.*

Applicants respectfully traverse. Amended claims 8 and 9 recite "screening for a test compound or a salt thereof that changes a binding property of lysophosphatidic acid or a salt thereof to an EDG-2 receptor or a salt thereof; wherein the EDG-2 receptor comprises the amino

acid sequence of SEQ ID NO: 1 or an amino acid sequence with at least 95% homology to the amino acid sequence of SEQ ID NO:1; comprising the steps of: a) bringing into contact the lysophosphatidic acid, the EDG-2 receptor, and the test compound; b) measuring the binding property of the lysophosphatidic acid and the EDG-2 receptor; c) determining whether the test compound changes the binding property of the lysophosphatidic acid and the EDG-2 receptor; and d) determining whether the test compound inhibits mesangial cell growth." Claims 8 and 9 do not refer to the treatment of kidney diseases but are drawn to a method for screening for a compound that requires the step of "determining whether the test compound inhibits mesangial cell growth." As shown in Example 1 of the specification, the EDG-2 receptor is expressed in mesangial cells and the specification teaches that the "EDG receptor family . . . are involved in renal diseases . . . mediated by regulating proliferation of mesangial cells . . ." (Example 1). As discussed below, one skilled in the art would know how to determine inhibition of mesangial cell growth. Thus, one skilled in the art would have known how to screen for "a compound or a salt thereof that changes a binding property of lysophosphatidic acid or a salt thereof to an EDG-2 receptor or a salt thereof" as recited in the claims. Thus, Applicants assert that claims 8 and 9 are fully enabled.

New claim 24, however, recites "[t]he method of claim 8, further comprising the step of determining whether the test compound is useful for treating diabetic nephropathy, chronic renal failure, nephritis, glomerulonephritis, interstitial renal disease or renal edema." The standard for enablement is whether one skilled in the art could practice the claimed invention using the teaching of the specification and information known in the art without undue experimentation. *See, e.g.,* MPEP § 2164; *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). Thus, the enablement standard permits one skilled in the art to use not just the specification, but also the relevant

knowledge and skill in the art, in order to practice the claimed invention. Prior to the earliest filing date of the instant application, the art recognized that (1) LPA binds to the EDG-2 receptor, (2) LPA is involved in mesangial cell growth, and (3) mesangial expansion is associated with renal disease.

Applicants herein present the following representative references to illustrate the state of the art regarding LPA, mesangial cell growth, and their relevance to kidney diseases known at the time of the earliest filing date of the instant application (December 12, 2002):

a. Gaits et al., "Dual Effect of Lysophosphatidic Acid on Proliferation of Glomerular Mesangial Cells" *Kidney Int* 51(4):1022-1027 (1997) (copy enclosed)

b. Inoue et al., "Lysophosphatidic Acid and Mesangial Cells: Implications for Renal Disease" *Clin Sci (Lond)* 96(4):431-436 (1999) (submitted in the IDS of June 6, 2005)

c. Inoue, "LPA as a Determinant of Mesangial Growth and Apoptosis" *Semin Nephrol.* 22(5):415-422 (2002) (copy enclosed)

d. Lehmann et al., "Molecular Mechanism of Diabetic Nephropathy" *Clin Chim Acta* 297(1-2):135-44 (2000) (copy enclosed)

e. Steffes et al., "Mesangial Expansion as a Central Mechanism for Loss of Kidney Function in Diabetic Patients" *Diabetes* 38(9):1077-1081 (1989) (copy enclosed)

At the time of the earliest filing date of the instant application, it was known that "Edg-2, -4, and -7 predominantly use LPA as their endogenous ligands." Inoue 2002 at page 415, right column. It was also known that LPA promotes mesangial cell survival "by the induction of antiapoptotic signaling pathways mediated by Bcl-xL." *Id.* at page 420, Figure 5. Furthermore, other experiments had shown that "LPA stimulates mesangial cell proliferation, which is associated with activation of mitogen-activated protein kinases (MAP kinases) p42 and p44." Gaits at page 1022, right column. According to Steffes, "[a] typical biopsy in a patient with advanced diabetic nephropathy and declining kidney function will contain hyalinized, obliterated

glomeruli and open glomeruli with marked mesangial expansion." Steffes at page 1078, right column. *See also* Lehmann, abstract. Steffes, in fact, stated that "[their] work with the structural and functional natural history of diabetic nephropathy has identified the expansion of the mesangium and the reduction in peripheral capillary surface as constituting the mechanism leading to the demise in kidney function." Steffes at page 1078, right column. Thus, the art recognized the relationship between LPA and mesangial growth in addition to the relationship between mesangial growth and renal disease.

Furthermore, the instant application shows that human normal mesangial cells express the Edg-2 receptor, *see* Example 1, and that Edg-2 receptor expression in the kidney increases in Wistar fatty rats compared to Wistar lean rats, *see* Example 2 and Figure 1. One skilled in the art would recognize the Wistar fatty rat as a model of diabetic nephropathy. For example, as early as 1992, those skilled in the art recognized that "[k]idney disease appears to be a common complication of spontaneous diabetes mellitus in many animal species." Kimmel at page 146, right column. Specifically, it was well known that "[r]enal disease in Wistar fatty rats is marked by onset of albuminuria and decreased glomular filatration rate at 20 weeks of age." *Id.* at page 144, left column. In addition, [g]lomerular and senagial volume and GBM surface area are significantly increased in 42-week-old Wistar fatty rats compared with lean controls." *Id.*

Thus, taking together the state of the art at the time of the earliest filing date of the instant application and the disclosure of the instant application, one skilled in the art would recognize the correlation between LPA, Edg-2 receptor, mesangial growth, and kidney disease. Accordingly, one skilled in the art would understand how to "screen[] for a test compound or a salt thereof that changes a binding property of lysophosphatidic acid or a salt thereof to an EDG-2 receptor or a salt thereof . . . [and] determining whether the test compound is useful for treating

diabetic nephropathy, chronic renal failure, nephritis, glomerulonephritis, interstitial renal disease or renal edema."

Despite the Office's contention that "no indication is presented [in the instant application] with regard to the type of cells that have a higher expression of E[D]G-2 receptors, it may be any of the cell types that constitute the tissue might have contributed to the result disclosed," one skilled in the art would reasonably expect that mesangial cell expansion was associated with the renal disease in this rat model as it is in humans. Accordingly, one skilled in the art could reasonably conclude that the increased Edg-2 expression observed in the fatty rats correlated with mesangial expansion.

For at least these reasons, Applicants respectfully request the withdrawal of the rejection.

II. Rejection of claims 8 and 9 under 35 U.S.C. § 112, Second Paragraph

Claims 8 and 9 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Office alleged that term "'substantially the same' with regard to the amino acid Seq. Id.: 1 . . . is indefinite because there is no quantitative limitation as to what its meaning is and therefore the meets [sic] and bounds of the claim could not be assessed." Action at page 5. The Office also stated that "with regard to Seq. Id. No.: 1, the claim refers to 'its partial peptide', which again is lacking any upper or lower limitation so that the meets [sic] and bounds of the claim could not be established." *Id.*

Applicants respectfully traverse. Claims 8 and 9 now recite "at least 95% homology to the amino acid sequence of SEQ ID NO:1." Support for this amendment is noted above. Accordingly, Applicants respectfully request that the Office withdraw the rejection.

III. Rejection of Claims 8 and 9 under 35 U.S.C. § 102(b)

Claims 8 and 9 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Erickson et al. (U.S. Patent 6,485,922). According to the Office, Erikson et al. teaches:

> [A] method for identifying compounds which modulate the activity of any of the EDG receptors, comprising the steps of exposing a compound and LPA to the EDG-2 receptor coupled to a response pathway, under conditions and for a time sufficient to allow interaction of LPA with the EDG-2 receptor and an associated response through the pathway, and b) detecting an increase or a decrease in the stimulation of the response pathway, relative to the absence of the tested compound (col. 6 from line 28 to col. 7, line 42).

Action at page 6. The Office also states that "Seq. Id No: 20, [of Erikson et al.] is identical to Seq Id. No.: 1 of the instant application." *Id.* The Office further states that "[s]ince the detection of any activation of the EDG-2 receptor is necessarily linked to the binding of the LPA to the EDG-2 receptor, the limitations of [] claim[s] 8 and 9 [are] present in Erickson et al." *Id.* Applicants respectfully traverse. Claims 8 and 9 are now drawn to "screening for a test compound or a salt thereof that changes a binding property of lysophosphatidic acid or a salt thereof to an EDG-2 receptor or a salt thereof; wherein the EDG-2 receptor comprises the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence with at least 95% homology to the amino acid sequence of SEQ ID NO:1; comprising the steps of: a) bringing into contact the lysophosphatidic acid, the EDG-2 receptor, and the test compound; b) measuring the binding property of the lysophosphatidic acid and the EDG-2 receptor; c) determining whether the test compound changes the binding property of the lysophosphatidic acid and the EDG-2 receptor; and d) determining whether the test compound inhibits mesangial cell growth."

According to the M.P.E.P., "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." M.P.E.P. § 2131 (citing *Verdegaal Bros.* v. *Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987)). Erikson et al. does not teach each and every element of the instant claims. Specifically, nowhere does Erikson et al. teach or suggest a step for determining whether a test compound inhibits mesangial cell growth. Therefore, Erikson et al. does not anticipate the claims and withdrawal of the rejection is respectfully requested.

IV. Rejection of Claims 8 and 9 under 35 U.S.C. § 102(e)

Claims 8 and 9 were rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by Miller et al. (U.S. Patent 6,875,757). The Office alleged that "Miller et al. teach [a] method of modulating LPA activity on an LPA receptor which includes providing a compound of the present invention which has activity as an LPA receptor antagonist and contacting an LPA receptor with the compound under conditions effective to inhibit LPA-induced activity of the LPA receptor (col. 8, lines 10-40)." Action at page 7.

Applicants respectfully traverse. Independent claims 8 and 9 are now drawn to "screening for a test compound or a salt thereof that changes a binding property of lysophosphatidic acid or a salt thereof to an EDG-2 receptor or a salt thereof; wherein the EDG-2 receptor comprises the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence with at least 95% homology to the amino acid sequence of SEQ ID NO:1; comprising the steps of: a) bringing into contact the lysophosphatidic acid, the EDG-2 receptor, and the test compound; b) measuring the binding property of the lysophosphatidic acid and the EDG-2 receptor; c) determining whether the test compound changes the binding property of the lysophosphatidic

acid and the EDG-2 receptor; and d) determining whether the test compound inhibits mesangial cell growth." Miller et al., however, does not teach or suggest a compound that inhibits mesangial cell growth. While Miller et al. discusses antagonists of the LPA receptor, Miller et al. broadly discusses modulating the LPA receptor for the treatment of wounds, *see* col. 31, line 37 – col. 32, line 49; enhancing cell proliferation, *see* col. 8 lines 10-21; and treating cancer, see col. 8, lines 22-26, but nothing regarding inhibiting mesangial cell growth. Furthermore, Miller et al. provides a table listing human tissues with the highest expression of various EDG receptors. This table, however, makes no mention of the kidney. Instead, Miller et al. notes that cardiovascular tissue, the CNS, gonadal tissue, and gastrointestinal tissue have the highest EDG-2 receptor expression. *See* table 2 at col. 5. Because Miller et al. does not teach or suggest a compound that inhibits mesangial cell growth as recited in claims 8 and 9, or a compound that may be useful for treating the renal diseases recited in claim 24, Miller et al. does not anticipate the claims. Applicants therefore respectfully request the withdrawal of the rejection.

Conclusion

In view of the foregoing amendments and remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: November 26, 2007

By: _____

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Kidney International, Vol. 51 (1997), pp. 1022–1027

Dual effect of lysophosphatidic acid on proliferation of glomerular mesangial cells

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Dual effect of lysophosphatidic acid on proliferation of glomerular mesangial cells. Among the variety of factors able to contribute to mesangial hypertrophy by altering mesangial cell growth, lysophosphatidic acid (LPA) is the focus of increasing attention. It is produced in plasma following platelet activation, as well as by mesangial cells stimulated by secretory phospholipase A_2. As mitogenic/antimitogenic properties of LPA are already described in a variety of cells, knowledge of its specific actions on mesangial cells is of potential interest regarding the pathophysiology of glomerulus damage *in situ*. We tested the effect of LPA on cultured rat mesangial cell growth. At 10 to 20 μM, LPA stimulated thymidine incorporation as well as phosphorylation of mitogen-activated protein kinases (MAP-kinases) p42-p44 in dose- and time-dependent manner, which demonstrated a positive effect on cell proliferation. However, higher concentrations of LPA (100 μM) were unable to stimulate thymidine incorporation and partly inhibited the proliferative effect as well as p42-p44 phosphorylation evoked by serum. Finally, whereas lysophosphatidylcholine (10 to 20 μM) was lytic for mesangial cells, no cell lysis could be detected at the highest concentrations of LPA. Taken together, these results suggest that LPA exerts a dual effect on mesangial cell proliferation, which could be due to activation of distinct specific signaling pathways, in dose-dependent fashion. Specific actions of LPA able to modify mesangial cell proliferation in a positive or negative manner are also likely to influence the pathophysiological processes involved in the progression of glomerulosclerosis in the kidney.

Mesangial expansion and mesangial cell proliferation usually precede the development of glomerular sclerosis [1]. A number of factors (growth factors, cytokines or vasoactive peptides) alter mesangial cell growth *in vitro*, which may contribute to mesangial hypertrophy *in vivo* [2–7]. Among the variety of cellular lipids, lysophosphatidic acid (LPA), a structurally simple phospholipid (Fig. 1), is the focus of increasing attention [8, 9]. LPA rapidly produced in the plasma following activation of platelets exerts growth-factor-like activity [9, 10], and was recently reported to evoke contractility and Ca^{2+} mobilization in mesangial cells [11]. In addition, LPA is produced by mesangial cells stimulated by exogenous group II phospholipase A_2 [12], this secretory enzyme being considered to be crucial in the initiation and propagation of inflammation by mesangial cells [13, 14]. In a recent hypothesis, we suggested that LPA can be generated upon hydrolysis of phosphatidic acid (note structure in Fig. 1) by secretory phospholipase A_2, *via* a specific mechanism involving membrane shedding, which points to possible local production of LPA in the vicinity of cells during inflammatory processes [15].

Because of the potential roles of this lipid mediator during mesangial expansion in chronic and progressive glomerulonephritis, we tested the effect of LPA on cultured mesangial cell growth. In the present study we demonstrate that LPA stimulates mesangial cell proliferation, which is associated with activation of mitogen-activated protein kinases (MAP kinases) p42 and p44. Moreover, these results are consistent with a dual effect of LPA on mesangial cells, higher concentrations of LPA exhibiting an antagonistic effect on proliferation.

Methods

Materials

Anti-MAP kinase antibody and CDP-Star chemiluminescent reagent were from New England Biolabs Ltd. (Beverly, MA, USA). [^{3}H]thymidine (20 Ci/mmol) was from Du Pont NEN (Les Ulis, France). Protein molecular mass standards were from Pharmacia (Uppsala, Sweden). Fetal calf serum (FCS) was from Gibco BRL (Paisley, Scotland, UK). Multiwells and culture flasks were from Falcon Plastic (Paisley, Scotland, UK). PVDF membrane was from Bio-Rad (Hercules, CA, USA). Lysophosphatidic acid, lysophosphatidylcholine, lysophosphatidylinositol, insulin, D-valine medium, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), RPMI 1640, trypsin, collagenase, *in vitro* toxicology assay kit (lactate dehydrogenase based) and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of rat renal glomeruli and culture of mesangial cells

Renal glomeruli were isolated from kidneys of two months old male Wistar rats weighing 150 to 200 g. Most of the procedure was adapted from Striker and Striker [16] as described [17]. Briefly, the kidneys were removed from their capsule and the cortex fragments (3 to 4 mm^3) were submitted to four repeated enzymatic digestions with collagenase at 37°C in phosphate-buffered saline (PBS) solution. The glomeruli, purified by successive mechanical sieving (100, 80 and 60 μm) were retained on the 80 and 60 μm pore stainless steal sieves. Glomerular mesangial cells were obtained from glomerulus preparations and cultured under standardized conditions at 37°C in a humidified 5% (vol/vol) CO_2 incubator, in base medium supplemented with 20% (vol/vol) decomplemented FCS plus 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Base medium was RPMI 1640.

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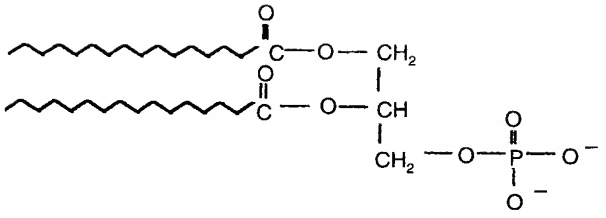
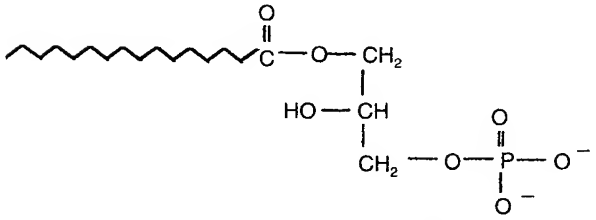


Phosphatidic acid (PA)



Lysophosphatidic acid (LPA)

Fig. 1. *Compared structures of phosphatidic and lysophosphatidic acids.*

Upon reaching confluence, cells were subcultured or harvested by using 0.125% (wt/vol) trypsin. Primary cultures were passaged after three to four weeks. In order to eliminate contamination by either epithelial or endothelial cells, all experiments were performed between the 15th and 25th passages. Mesangial cells were characterized by their morphological appearance in phase contrast and their biological and biochemical properties as described [17]: (1) positive staining with antibodies to myosin or vimentin, and negative staining with antibodies to factor VIII and to cytokeratins by standard immunofluorescence; (2) the ability to contract in response to 10^{-5} M angiotensin II; (3) an ability to grow in selective D-valine containing medium. Before these experiments, cells were made quiescent by serum starvation for 36 hours in RPMI as above with 0.5% (vol/vol) FCS.

[^{3}H]thymidine incorporation

Preconfluent quiescent mesangial cells cultured in 24-well tissue culture dishes were incubated with the growth stimulus to be tested. At the required time, [^{3}H]thymidine (0.25 mCi/ml) was added and the incubation continued for additional four hours. Cells were washed with PBS, lysed and precipitated with 5% (wt/vol) trichloroacetic acid (TCA). Precipitates were washed with absolute ethanol and then air dried. The TCA-precipitable material was dissolved in 0.5 M NaOH and the radioactivity was measured in triplicate with a liquid scintillation counter. The data were expressed as ratio (fold stimulation) of the mean control value.

MTT assay and cell lysis assay

Preconfluent quiescent mesangial cells were cultured in 12 well dishes and treated by the growth stimulus as indicated. The MTT assay was performed as described [18], in triplicate with OD_{570} read on a spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). Matching triplicate dishes were trypsinized and counted using a Coulter counter. The data were expressed as as ratio (fold stimulation) of the mean control value. For the cell lysis assay, lactate dehydrogenase activity was determined in both supernatants and cells using a kit from Sigma. The percentage of lysis was calculated from the ratio of enzyme activity in supernatant to enzyme activity in supernatant plus cells.

MAP-kinase phosphorylation assay

Phosphorylation of p42 and p44 MAP kinases (ERK1 and ERK2) was tested by means of an antibody raised against phosphorylated MAP kinases, which specifically detects p42 and p44 phosphorylated on the residue homologous of tyrosine 204 in p42 [19]. Briefly, after incubation cells were washed twice with PBS, then lysed with sodium dodecyl sulfate (SDS) sample buffer containing 62 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 50 mM dithiothreitol, and 0.1% (wt/vol) bromophenol blue. Homogenates were sonicated 10 seconds, clarified at 13,000 rpm two minutes, boiled two minutes, run onto SDS-PAGE, then electrotransferred to PVDF membrane. Membrane was blocked one hour by PBS buffer with 5% (wt/vol) nonfat dry milk, then incubated overnight with anti-phosphorylated MAP kinase antibody (dilution 1/1000), and another hour with alkaline phosphatase-conjugated anti-rabbit secondary antibody (dilution 1/4000). Detection was performed by means of CDP-Star chemiluminescent reagent. Each observation was confirmed at least twice.

Statistics

Results are expressed as mean ± SEM ($N = 3$) and are representative of two to six experiments, as indicated in the legends. Comparisons were made using Student's t-test; significance of results was determined by $P < 0.05$.

Results

Proliferation of mesangial cells under the effect of LPA

As shown in Figure 2A, incubation of mesangial cells with LPA during 24 hours resulted in a dose-dependent increase of thymidine incorporation, which was maximal at 20 μM LPA. Under these conditions, the stimulation ratio was 1.91 ± 0.18 (mean ± SEM, 7 independent experiments, $P < 0.01$). The full effect, however, was limited to LPA concentrations within 10 to 20 μM, stimulation of thymidine incorporation being not significant at 100 μM LPA. Under the latter conditions, whether LPA was neither lytic nor toxic for the cells was verified by Trypan blue exclusion. The effect of 20 μM LPA on thymidine incorporation was detectable at 12 hours, with a peak around 24 hours, and was kinetically comparable to that obtained with 5% FCS (data not shown). Stimulation of MTT conversion by LPA paralleled the effect on thymidine incorporation (Fig. 2B). Determination of cell number confirmed that low concentrations of LPA exerted a stimulatory effect on cell proliferation (Fig. 2C).

Effect of LPA on MAP-kinase tyrosine phosphorylation

To correlate the precedent effects of LPA on cell proliferation with biochemical events occurring downstream of putative LPA receptors, we tested the phosphorylation of p42 and p44 MAP kinases in stimulated cells. At 20 μM, LPA increased p42-p44 phosphorylation (Fig. 3). From quantitative data shown in Figure 4A, it appeared that LPA-induced phosphorylation of p42 and

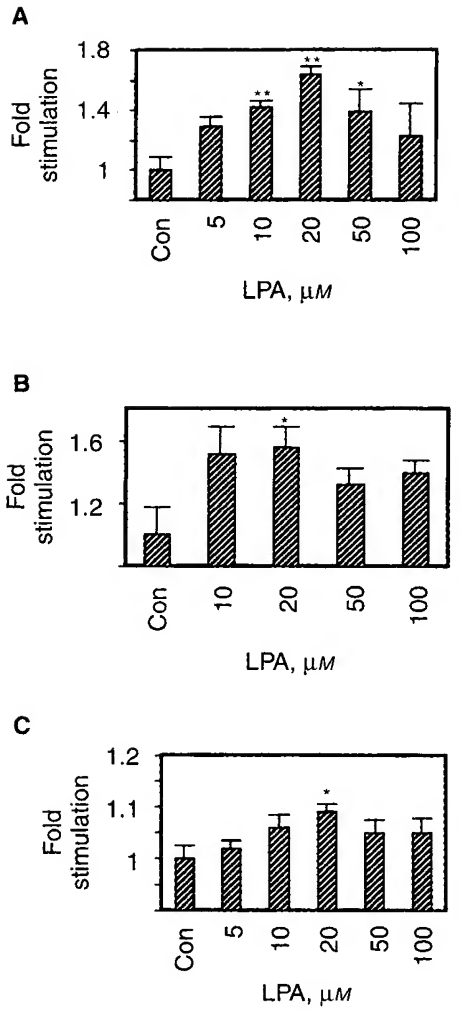


Fig. 2. *Effect of LPA on mesangial cell proliferation.* Subconfluent quiescent cells (15th to 20th passages) were incubated during 24 hours with various concentrations of LPA (5 to 100 μM) or vehicle as a control (con). Cells were then incubated with [^{3}H]thymidine (**A**), or MTT (**B**), or they were trypsinized for counting (**C**). Results (means ± SEM) are triplicate determinations from one experiment representative of 6, 2 and 3 independent experiments for A, B and C, respectively. $^*P < 0.05$; $^{**}P < 0.01$.

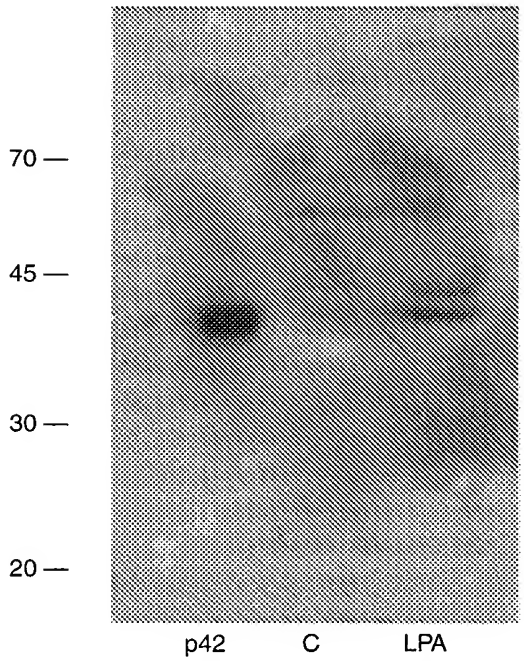


Fig. 3. *Effect of LPA on p42 and p44 MAP-kinase tyrosine phosphorylation.* Subconfluent quiescent cells (20th to 25th passages) were incubated during 10 minutes with vehicle only (C) or LPA (20 μM), run onto SDS-PAGE, electrotransferred and detected with anti-phosphorylated MAP-kinase antibody. Molecular mass standard positions (kDa) are indicated on the left. On the left lane, a standard of phosphorylated p42 was deposited onto the gel.

p44 paralleled the dose-dependent effect of LPA on thymidine incorporation, with a maximum at 10 to 20 μM LPA, and a decrease above. Cells stimulated by LPA exhibited a two-wave profile of MAP-kinase phosphorylation, a first peak appearing at 20 to 30 minutes, and a second peak, moderate and more sustained, occurring at 90 to 120 minutes (Fig. 4B). Furthermore, phosphorylation remained slightly detectable at 12 hours (not shown).

Inhibition of FCS-stimulated mesangial cell proliferation by high concentrations of LPA

Considering the slight effect of 100 μM LPA on mesangial cell proliferation, and since antimitogenic effects of LPA are also known, we tested the hypothesis of an inhibitory effect of LPA by incubating the cells with 5% (vol/vol) FCS in the presence of 100 μM LPA. FCS alone promoted a 7.5-fold stimulation of thymidine incorporation, which was decreased by 54% upon simultaneous addition of 100 μM LPA (Fig. 5A). In parallel, incubation of mesangial cells with 100 μM LPA and 5% FCS resulted in a decrease of the effect of FCS on MAP-kinase phosphorylation (Fig. 5B).

Determination of cell lysis in mesangial cells stimulated with LPA

To exclude the possibility that high concentrations of LPA might be deleterious to the cells owing to a detergent-like effect, lactate dehydrogenase activity was determined in supernatants of cells incubated for 24 hours with various concentrations of LPA. As shown in Table 1, no significant cell lysis could be detected even at 100 μM LPA. In fact, at all concentrations tested LPA reduced the release of lactate dehydrogenase occurring from mesangial cells incubated in a medium containing 0.5% serum. In contrast, lysophosphatidylcholine (LPC) exerted a lytic effect, which attained 43.6% at only 20 μM. In additional experiments dealing with shorter incubation times (1 hr), 50 μM LPC induced 73% lysis, whereas lysophosphatidylinositol was still more effective (61% and 85% lysis at 20 and 50 μM, respectively, data not shown).

Discussion

As a main conclusion of this study, a mitogenic effect of low concentrations of LPA could be demonstrated in cultured rat mesangial cells. In a study centered on the effects of phosphatidic acid, a similar action of LPA was observed in human mesangial cells, however, dose dependence was not investigated [20]. In fibroblasts, the intensity of the proliferative effect of LPA can reach the level attained with the serum itself [21]. In mesangial

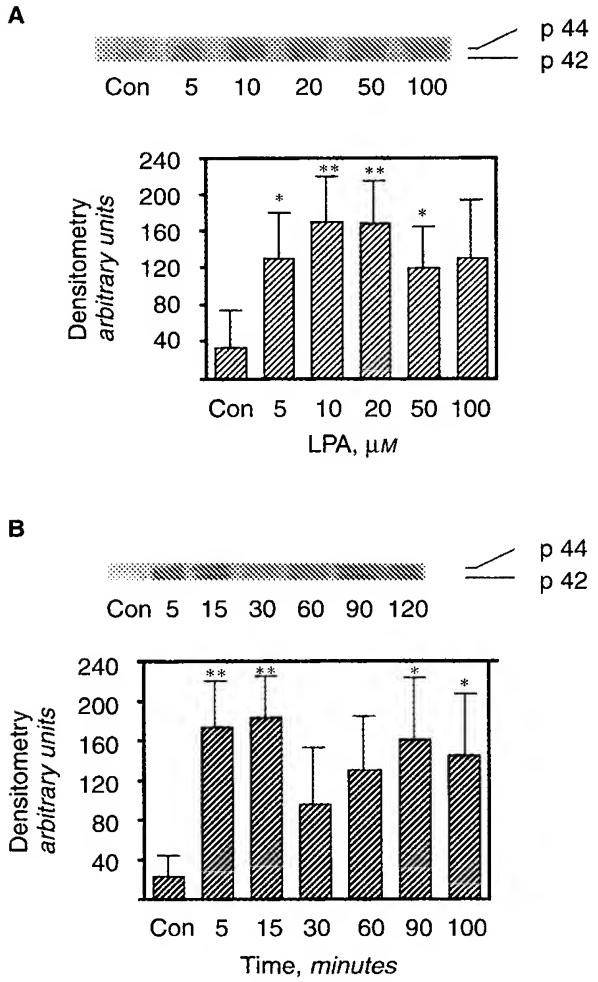


Fig. 4. *Time course and dose response of the effect of LPA on p42 and p44 MAP-kinase tyrosine phosphorylation.* Subconfluent quiescent cells (20th to 25th passages) were incubated with LPA. **A.** Dose response: cells were incubated for 15 minutes with indicated concentrations of LPA. **B.** Time course: cells were incubated for indicated times with 20 μM LPA. Upper part, SDS-PAGE, electrotransfer and detection with anti phosphorylated MAP-kinase antibody and chemiluminescence. Lower part, quantitation of p42 and p44 in data above after scanning (means ± SEM, 3 experiments). Abbreviation Con is controls without LPA. $*P < 0.05$; $**P < 0.01$.

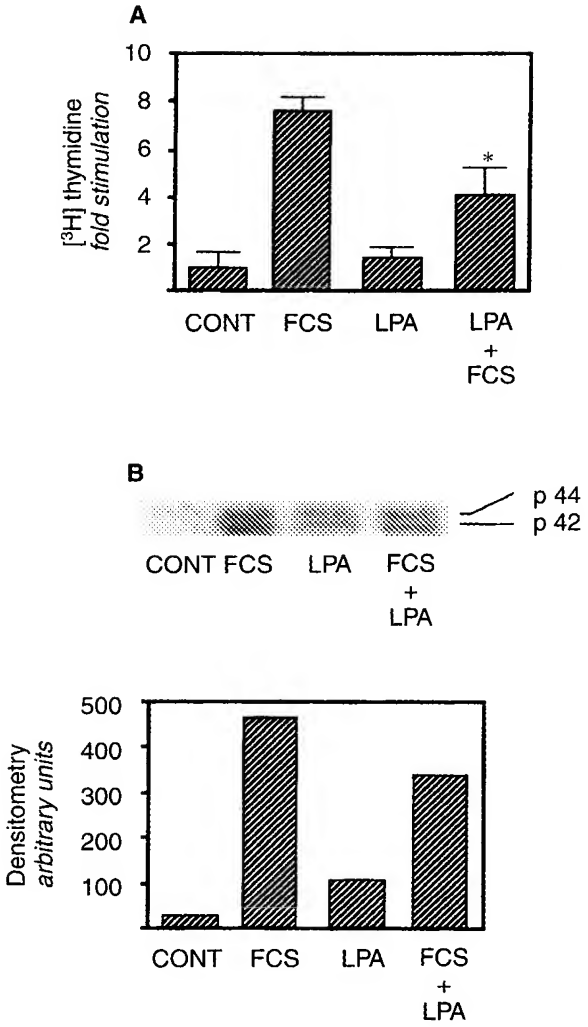


Fig. 5. *Effect of LPA on FCS stimulated [^{3}H]thymidine incorporation and p42-p44 MAP-kinase tyrosine phosphorylation.* **A.** Subconfluent quiescent cells (20th to 25th passages) were incubated for six hours with vehicle only (CONT), with 5% (vol/vol) FCS, with 100 μM LPA, or with both 5% FCS and 100 μM LPA, and then incubated for one hour with [^{3}H]thymidine. Data are means ± SEM of triplicate determinations and are representative of two independent experiments. $*P < 0.05$, comparison with FCS alone. **B.** Subconfluent quiescent cells were incubated for 15 minutes under conditions described in A. Upper part, SDS-PAGE, electrotransfer and detection with anti-phosphorylated MAP-kinase antibody and chemiluminescence. Lower part, quantitation of p42 and p44 in data above after scanning.

cells, the effect of LPA regarding thymidine incorporation was lower than that obtained with FCS, and was in the same range as that observed with other growth factors, like vasoactive peptides [4–6]. In many cases, these factors induce more hypertrophy than hyperplasia, or act as comitogens only [5–7, 22]. In addition to a stimulation of thymidine incorporation, LPA was responsible in mesangial cells for an increase of both MTT conversion and of cell number. In our opinion, this demonstrates that low concentrations of LPA alone exert an effective stimulatory effect on mesangial cell proliferation, suggesting a role of LPA as a progression factor, as has been suggested for insulin, platelet-derived growth factor, or insulin-like growth factor I [22, 23]. This is in accordance with data observed in fibroblasts, where LPA appears as a complete substitute to the stimulatory effect of serum, and is considered as a progression factor [21, 24]. Conversely, no major phenotypic modification suggesting hypertrophy was noticed during 48 hours of incubation with low doses of LPA.

A second conclusion concerns the mechanism of the mitogenic effect of LPA. This seems to involve phosphorylation of p42-p44 MAP kinases at tyrosine 204, which is known to be essential for the activation of MAP kinases [19]. Indeed, we could verify that LPA stimulated the myelin basic protein kinase activity of mesangial cells in a manner similar to that described for platelet-derived growth factor and angiotensin II (data not shown) [22]. Growth factor activation of p42-p44, when associated with effective proliferative responses, usually comprises a sustained phase

Table 1. Determination of cell lysis in mesangial cells incubated with lysophospholipids

Lysophospholipid	Cell lysis %	*P*
None	17.0 ± 1.9	
LPA 20 μM	10.8 ± 0.6	< 0.05[a]
LPA 50 μM	10.6 ± 0.2	< 0.05[a]
LPA 100 μM	11.3 ± 1.4	NS
LPC 10 μM	25.5 ± 4.0	< 0.001[a]; < 0.05[b]
LPC 20 μM	43.6 ± 2.3	< 0.001[a]; < 0.001[b]

Subconfluent quiescent cells (20 to 25 passages) were incubated during 24 hours with various concentrations of LPA or LPC or with vehicle. Cell lysis was determined by measuring lactate dehydrogenase activity in the supernatants. Data are means ± SEM (3 different incubations). Abbreviations are LPC, lysophosphatidylcholine; NS, nonsignificant.

[a] Compared to controls (no lysophospholipid)

[b] Compared to LPA 20 μM

during biphasic kinetic activation of MAP kinases [25, 26]. This fact, which was also observed with mesangial cells as a requirement for effective hyperplasic effects of various agonists [22], was observed herein with LPA. Activation of p42-p44 MAP kinases involves the sequential upstream stimulation of *ras* and *raf*, and has been recognized as an essential step required for cell division entry [25–29]. This pathway is activated by growth factors acting on membrane receptors with protein tyrosine kinase activity by mechanisms now clearly established [27–29]. In the case of agonists using receptors coupled to heterotrimeric G-proteins, such as LPA, the intermediate steps between receptor and *ras* are still unknown, although increasing evidence underlines a major role of protein tyrosine kinases acting downstream of the βγ-subunits of Gi [30–34]. From the parallelism between the biological effects reported herein and the phosphorylation level of p42-p44 MAP kinases, it thus appears rather obvious that similar if not identical transduction pathways could be used in mesangial cells by LPA under conditions leading to an increased proliferation.

The action of LPA on mesangial cells is in fact more complex, since the specificity of its mitogenic effect depends on the concentration used. Indeed, at 100 μM LPA was far less stimulatory, and was even able to partly inhibit the full effect of serum regarding thymidine incorporation as well as MAP-kinase phosphorylation. Since LPA did not appear to be toxic under these conditions, as shown by the absence of lysis, these data suggest that in mesangial cells the ultimate effect of high doses of LPA may be the sum of stimulatory and inhibitory actions. An antimitogenic effect of LPA was previously described in other cells [35, 36]. Especially in MDCK cells, LPA possesses mitogenic-antimitogenic effects in a dose-dependent manner [35].

Interestingly, the lack of mitogenic effects or inhibition by LPA of serum-stimulated proliferation were parallel to the variations in the level of p42-p44 phosphorylation. This gives further support to the view of a strong linkage between activation of the MAP-kinase pathway and stimulation of proliferation. Further studies will be necessary to clarify how LPA at high concentrations inhibits mesangial cell proliferation by interfering with signal transduction probably upstream of p42-p44 MAP kinases. The duality of LPA effect could be due to the presence of more than one type of receptors with different affinity for the phospholipid mediator [37]. For this or other reasons, LPA could activate additional signaling pathways, involving, for instance, *Jun* kinase (JNK). This cascade is stimulated by ceramides in mesangial cells, and it exerts opposing action on p42-p44 MAP kinases, leading to antiproliferative effects [28, 38]. This family of stress-activated kinases, as well as another family of p38/HOG1 kinases, seem able to induce apoptosis when activated by cytokines. It is thus tempting to suggest that mitogenic and antimitogenic effects of LPA responsible for apoptosis in a dose-dependent manner could finally positively or negatively influence renal scarring subsequent to glomerular inflammation [39]. Regarding the potential role of LPA in pathophysiology, complete knowledge of the action of this lipid mediator in mesangial cells seems of primary interest for a better understanding of the mechanisms underlying the progression of glomerulosclerosis.

Finally, it is interesting to compare the proliferative effect of LPA described herein to that exerted on mesangial cells by its putative precursor phosphatidic acid [20]. Although both compounds also act as growth factors on fibroblasts [24], the functional relationship between the two phospholipids is still unclear. Indeed, phosphatidic acid is known as an intracellular compound, whereas the extracellular actions of LPA are well documented [8, 9]. Since mesangial cells synthesize and secrete type II phospholipase A_2 under certain conditions, it is tempting to suggest that the extracellular effects of phosphatidic acid might involve its deacylation to LPA. However, internalization of phosphatidic acid resulting in an increased local concentration might also be involved in the mechanism of its biological effect, as suggested by Knauss, Jaffer and Abboud [20]. Further studies are required to quantitate the amounts of both phosphatidic acid and LPA produced by mesangial cells. This will be particularly important to assess the potential role of LPA as an autocrine factor regulating mesangial cell growth.

Acknowledgments

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References

1. STRIKER LJ, KILLEN RD, CHI E, SRIKER GE: The composition of glomerulosclerosis. I. Studies in focal sclerosis, crescentic glomerulonephritis, and membranoproliferative glomerulonephritis. *Lab Invest* 51:181–192, 1984
2. MENE P, SIMONSON MS, DUNN MJ: Physiology of the mesangial cell. *Physiol Rev* 69:1347–1424, 1989
3. DAVIES M: The mesangial cell: A tissue culture view. *Kidney Int* 45:320–327, 1994
4. GANZ MB, PERFETTO MC, BORON WF: Effect of mitogens and other agents on rat mesangial cell proliferation, pH and Ca^{2+}. *Am J Physiol* 28:F269–F278, 1990
5. RAY PE, AGUILERA G, KOPP JB, HORIKOSHI S, KLOTMAN PE: Angiotensin receptor-mediated proliferation of cultured human fetal mesangial cells. *Kidney Int* 40:764–771, 1991
6. WOLTHUIS A, BOES A, RODEMANN HP, GROND J: Vasoactive agents affect growth and protein synthesis of cultured mesangial cells. *Kidney Int* 41:124–131, 1992
7. ANDERSON PW, DO YS, HSUEH WA: Angiotensin II causes mesangial cell hypertrophy. *Hypertension* 21:29–35, 1993

8. MOOLENAAR WH: Lysophosphatidic acid, a multifunctional phospholipid messenger. *J Biol Chem* 270:12949–12952, 1995
9. JALINK K, HORDIJK PL, MOOLENAAR WH: Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. *Biochim Biophys Acta* 1198:185–196, 1994
10. EICHHOLTZ T, JALINK K, FARRENFORT I, MOOLENAR WH: The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* 291:677–680, 1993
11. INOUE CN, FORSTER HG, EPSTEIN M: Effects of lysophosphatidic acid, a novel lipid mediator, on cytosolic Ca^{2+} and contractility in cultured rat mesangial cell. *Circ Res* 77:888–896, 1996
12. WADA A, SUGIURA T, ARAI M, TOJO H, OKAMOTO M, FUJIWARA Y, UEDA N, KAMADA T: Group II phospholipase A_2 promotes mesangial cell proliferation via phospholipids. (abstract) *J Am Soc Nephrol* 5:704, 1994
13. PFEILSCHIFTER J, SCHALKWIJK C, BRINER VA, VAN DEN BOSCH H: Cytokine-stimulated secretion of group II phospholipase A_2 by rat mesangial cells. *J Clin Invest* 92:2516–2523, 1993
14. SUGIURA T, WADA A, ITOH T, TOJO H, OKAMOTO M, IMAI E, KAMADA T, UEDA N: Group II phospholipase A_2 activates mitogen-activated protein kinase in cultured rat mesangial cells. *FEBS Lett* 370:141–145, 1995
15. FOURCADE O, SIMON MF, VIODÉ C, RUGANI N, LEBALLE F, RAGAB A, FOURNIÉ B, SARDA L, CHAP H: Secretory phospholipase A_2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* 80:919–927, 1995
16. STRIKER GE, STRIKER LJ: Glomerular cell culture. *Lab Invest* 53:122–131, 1985
17. SALLES JP, GAYRAL-TAMINH M, FAUVEL J, DELOBBE I, MIGNON-CONTÉ M, CONTÉ JJ, CHAP H: Sustained effect of angiotensin II on tyrosine phosphorylation of annexin I in glomerular mesangial cells. *J Biol Chem* 268:12805–12811, 1993
18. MOSMANN T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assays. *J Immunol Meth* 65:55–63, 1983
19. PAYNE DM, ROSSOMANDO AJ, MARTINO P, ERICKSON AK, HER JH, WEBER MJ, STURGILL TW: Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J* 10:885–892, 1991
20. KNAUSS TC, JAFFER FE, ABBOUD HE: Phosphatidic acid modulates DNA synthesis, phospholipase C, and platelet-derived growth factor mRNAs in cultured mesangial cells. Role of protein kinase C. *J Biol Chem* 265:11457–11463, 1990
21. VAN CORVEN EJ, GROENINK A, JALINK K, EICHHOLTZ T, MOOLENAAR WH: Lysophosphatidate-induced cell proliferation: Identification and dissection of signaling pathways mediated by G proteins. *Cell* 59:45–54, 1989
22. HUWILER A, STABEL S, FABBRO D, PFEILSCHIFTER J: Platelet-derived growth factor and angiotensin stimulate the mitogen-activated protein kinase cascade in renal mesangial cells: Comparison of hypertrophic and hyperplasic agonists. *Biochem J* 305:777–784, 1995
23. DOI T, STRIKER LJ, ELLIOTT SJ, CONTI FG, STRIKER GE: Insulin-like growth factor is a progression factor for human mesangial cells. *Am J Pathol* 134:395–404, 1989
24. VAN CORVEN EJ, VAN RIJSWIJK, JALINK K, VAN DER BEND RL, VAN BLITTERSWIJK WJ, MOOLENAAR WH: Mitogenic action of lysophosphatidic acid and phosphatidic acid on fibroblasts. Dependence on acyl-chain length and inhibition by suramin. *Biochem J* 281:163–169, 1992
25. KAHAN C, SEUWEN K, MELOCHE S, POUYSSÉGUR J: Coordinate biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts. *J Biol Chem* 267: 13369–13375, 1992
26. PAGES G, LENORMAND P, L'ALLEMAIN G, CHAMBARD JC, MELOCHE S, POUYSSÉGUR J: Mitogen-activated protein kinases p42 mapk and p44 mapk are required for fibroblast proliferation. *Proc Natl Acad Sci USA* 90:8319–8323, 1993
27. NISHIDA E, GOTOH Y: The MAP-kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem Sci* 18:128–131, 1993
28. BOKEMEYER D, SOROKIN A, DUNN MJ: Multiple intracellular MAP kinase signaling cascades. *Kidney Int* 49:1187–1198, 1996
29. COBB MH, GOLDSMITH EJ: How MAP kinases are regulated. *J Biol Chem* 270:14843–14846, 1995
30. HOWE LR, MARSHALL CJ: Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring $p21^{ras}$ and $p74^{raf-1}$. *J Biol Chem* 268:20717–20720, 1993
31. HORDIJK PL, VERLAAN I, VAN CORVEN EJ, MOOLENAAR WH: Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of MAP kinase is mediated by the Gi-$p21^{ras}$ pathway. *J Biol Chem* 269:645–651, 1994
32. KOCH WJ, HAWES BE, ALLEN LF, LEFKOWITZ RJ: Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G\beta\gamma$ activation of $p21^{ras}$. *Proc Natl Acad Sci USA* 91:12706–12710, 1994
33. VAN BIESEN T, HAWES BE, LUTTRELL DK, KRUEGER KM, TOUHARA K, PORFIRI E, SAKAUE M, LUTTRELL LM, LEFKOWITZ RJ: Receptor-tyrosine-kinase- and $G\beta\gamma$-mediated MAP kinase activation by a common signalling pathway. *Nature* 376:781–784, 1995
34. GAITS F, LI RY, BIGAY J, RAGAB A, RAGAB-THOMAS JMF, CHAP H: G-protein $\beta\gamma$ subunits mediate specific phosphorylation of the protein-tyrosine phosphatase SH-PTP1 induced by lysophosphatidic acid. *J Biol Chem* 271:20151–20155, 1996
35. BASHIR N, KUHEN K, TAUB M: Phospholipids regulate growth and function of MDCK cells in hormonally defined serum free medium. *In Vitro Cell Develop Biol* 28A:663–668, 1992
36. TIGYI G, DYER DL, MILEDI R: Lysophosphatidic acid possesses dual action in cell proliferation. *Proc Natl Acad Sci USA* 91:1908–1912, 1994
37. LILIOM K, MURAKAMI-MUROGUSHI K, KOBAYASHI S, MUROFUSHI H, TIGYI G: Xenopus oocytes express multiple receptors for LPA-like lipid mediators. *Am J Physiol* 270:C772–C777, 1996
38. XIA Z, DICKENS M, RAINGEAUD J, DAVIS RJ, GREENBERG ME: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270:1326–1331, 1995
39. SAVILL J, MOONEY A, HUGHES J: Apoptosis and renal scarring. *Kidney Int* 49(Suppl 54):S14–S17, 1996

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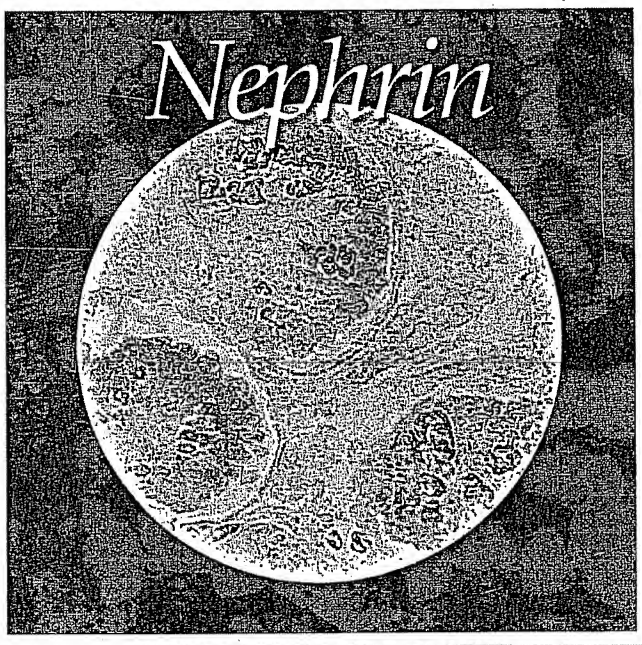
SEMINARS IN NEPHROLOGY

A. Kurtzman, MD



Naturally Occurring Cardiovascular and Renal Mediators

Murray Epstein, MD

Guest Editor

W.B. SAUNDERS

LPA as a Determinant of Mesangial Growth and Apoptosis

By Chiyoko N. Inoue

Mesangial cell proliferation is a prominent feature of progression in many forms of renal diseases, including immunoglobulin A nephropathy, lupus nephritis, hemolytic uremic syndrome, and diabetic nephropathy. Platelet-derived growth factor (PDGF) has received much attention as the major mediator of mesangial cell proliferation by autocrine/paracrine mechanisms involving up-regulation of mesangial PDGF and its receptor on mesangial cells. In this review, we wish to spotlight lysophosphatidic acid (LPA), which in combination with PDGF, undoubtedly plays a key role as an autocrine and paracrine mediator in regulating mesangial cell growth. We not only showed that PDGF acts as a bimodal molecule for mesangial cells, inducing mesangial cell proliferation and death simultaneously, but also showed that LPA is a survival factor suppressing PDGF-induced mesangial cell death, thereby remarkably enhancing mesangial mitogenic response by PDGF. We believe that a better understanding of the mechanisms of mesangial cell proliferation by the combined action of PDGF and LPA could lead to novel diagnostic as well as therapeutic strategies, and thus help to better control proliferative glomerulonephritis.

RECENTLY, MUCH attention has been focused on the potential role of lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol-3-phosphate) in the pathologic process of various human inflammatory or malignant diseases. LPA was first identified in serum by Schumacher et al[1] in 1979 as a substance that potently stimulates aggregation of platelets. Although this report did not get much attention at the time, LPA later re-emerged and is now established as an authentic phospholipid intercellular signaling molecule that has its own specific cell-surface receptor.[2,3] LPA is a member of the lysoglycerol-containing phospholipid subfamily, which can be generated by activated platelets or in membranes of injured cells.[2] So far, accumulation of LPA has been shown in serum, ascitic fluid from patients with intraperitoneal malignancies, and in inflammatory exudates in micromolar concentration sufficient to evoke tissue reactions.[2] In plasma, LPA is almost entirely bound to proteins such as albumin and gelsolin.[4] Many reports have also explored various cellular responses of LPA and the number and diversity continue to grow. The effects of LPA on cells include growth, apoptosis, shape changes, tumor invasion, and wound healing.[5]

The best known source of extracellular LPA are platelets, which release LPA after activation.[6] LPA is also generated in activated plasma membrane when phospholipid symmetry is destroyed,[7] where LPA is produced from precursor phospholipids by phospholipase C (PLC)- as well as PLD-dependent libration of phosphatidic acid and its conversion to LPA by the group II PLA_2.[8,9] Stimulation of group II PLA_2 in inflammatory processes via interleukin-1β or tumor necrosis factor-α in glomerulonephritis has been well documented.[10,11] Recently, LPA has been reported to be formed from low-density lipoproteins as a result of mild oxidative stress of low-density lipoproteins. LPA has indeed been identified in athelosclerotic plaques, where LPA is suggested to trigger platelet activation and stimulate the formation of endothelial stress fiber and gaps, promoting endothelial permeability.[12,13] Oxidized low-density lipoprotein has also been identified in glomeruli in patients with focal segmental glomerulosclerosis, suggesting a role in renal glomerular diseases.[14]

Several subtypes of LPA-receptors have been identified as a family of closely related G-protein–coupled receptors. Among 7 family members of lysophospholipid receptors named for the endothelial differentiation gene (Edg) receptors, Edg-2, -4, and -7 predominantly use LPA as their endogenous ligands. Edg receptors are expressed in most mammalian cells and tissues, each subtype having a distinct distribution pattern, raising the possibility of tissue-specific biologic roles of LPA.[15]

The focus of this article is the role of LPA in regulating renal mesangial cell growth and apoptosis. The experimental data were obtained in vitro by manipulating mesangial cell growth and death

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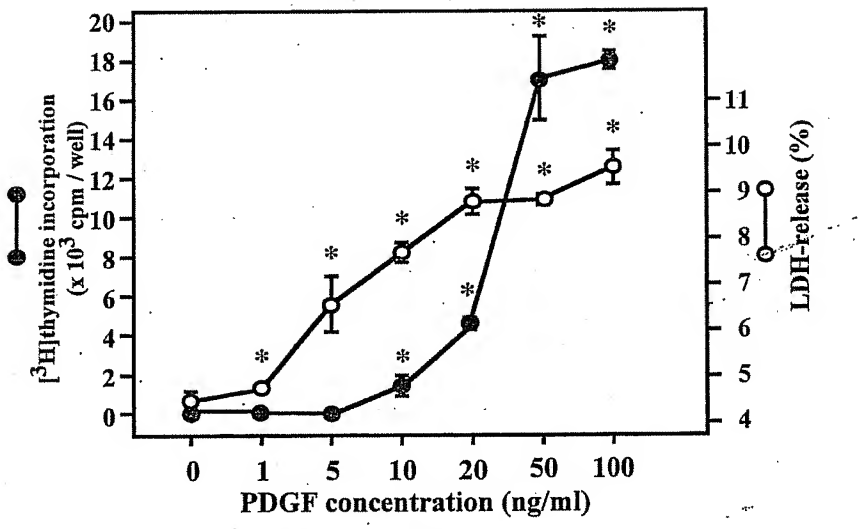

Fig 1. Dose-dependent stimulation of DNA synthesis and LDH release by PDGF. Primary cultured rat mesangial cells were grown to subconfluence and rendered quiescent in RPMI 1640/0.1% bovine serum albumin/0.5% fetal bovine serum for 2 days. Cells were stimulated with the indicated dose of PDGF-BB for 24 hours, and the DNA synthesis was assessed by measuring [^{3}H]thymidine uptake for the last 6 hours (n = 3 wells). In parallel, LDH activities in the cell culture supernatant were measured and expressed as a percentage of total LDH activity in the corresponding culture dish (n = 3 wells). Under the stimulation of PDGF, both mesangial DNA synthesis and death occur simultaneously. *$P < .05$ versus quiescent cells. Reprinted with permission from Inoue et al: Bimodal effects of platelet-derived growth factor on rat mesangial cell proliferation and death, and the role of lysophosphatidic acid in cell survival. Clin Sci (Colch) 101:11-19, 2001.[20]

using PDGF and LPA in primary cultured rat mesangial cells.[16] Primary cultured mesangial cells are an established experimental system that faithfully reflects physiologic and pathophysiolgic changes of glomerular mesangium in vivo, including contractility, ability to synthesize matrix, and production of growth factors and cytokines.[17] By way of introduction, a brief description is given of the effect of PDGF on cultured mesangial cells.

BIMODAL EFFECTS OF PDGF ON MESANGIAL CELL PROLIFERATION AND DEATH

Over the past 10 years, the important mitogenic role of PDGF in mesangial cell proliferation has been well established. PDGF is a polypeptide growth factor that is produced within glomeruli by platelets as well as by activated macrophage/monocytes or mesangial cells themselves, and exhibits a potent mitogenic activity autocrinely and paracrinely for mesangial cells.[18,19] However, recent studies from our laboratory have shown that PDGF is a double-edged sword for mesangial cells. Although PDGF induces proliferation, it also simultaneously evokes mesangial cell death.[20] Fig 1 shows simultaneous mesangial mitogenic and death responses expressed by [^{3}H]thymidine incorporation and lactate dehydrogenase (LDH) leakage into medium, respectively. Although PDGF dose-dependently (10–50 ng/mL) induces mesangial DNA synthesis, mesangial cell death assessed by LDH release is also dose-dependently (5–100 ng/mL) elevated. A low concentration of PDGF (5 ng/mL) only causes LDH release in the absence of a significant increase in DNA synthesis. By this experiment, it became clear that when mesangial cells are stimulated by PDGF, both mesangial cell proliferation and death occur side by side, and that PDGF even acts as a cytotoxic factor at low concentrations.

Cell death is largely divided into 2 categories, apoptosis and necrosis. Apoptosis is suicidal death of cells triggered by either internal or external physiologic signals. Necrosis is cell death triggered by external insults such as oxygen deprivation or exposure to cytotoxic chemical agents.[21] To characterize the death mechanism by PDGF, we used standard apoptosis-related assays in PDGF-treated mesangial cells, including electron microscopic observation and DNA fragmentation assay. Treat-

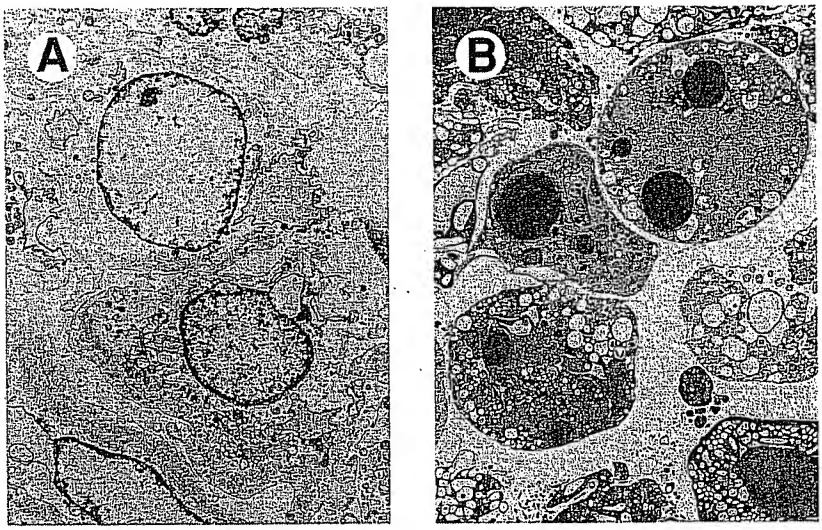

Fig 2. Apoptotic mesangial cell death by PDGF. Quiescent mesangial cells were stimulated with PDGF (50 ng/mL for 24 hr) and then processed for electron microscopy. (B) In PDGF-stimulated and detached cells, but not in (A) quiescent and adherent cells, typical morphologic changes, including cellular shrinkage, nuclear chromatin condensation, and fragmentation and multiple cytoplasmic vacuole formation, are prominent. Magnification: ×2,690. Reprinted with permission from Inoue et al: Bimodal effects of platelet-derived growth factor on rat mesangial cell proliferation and death, and the role of lysophosphatidic acid in cell survival. Clin Sci (Colch) 101:11-19, 2001.[20]

ment of rat mesangial cells with PDGF induces typical morphologic changes of apoptosis, including nuclear condensation, chromatin fragmentation cellular shrinkage, and the formation of multiple cytoplasmic vacuoles (Fig 2). We also identified that dying PDGF-treated mesangial cells showed DNA ladder formation and enhanced p53 expression, which have been reported to be involved in the apoptotic process of cultured mesangial cells.[20,22] Given that PDGF-induced cell death was completely suppressed by protein synthesis inhibitor, cycloheximide, our data suggest that death is mainly mediated by apoptosis.

EFFECT OF LPA ON MESANGIAL SURVIVAL AND PROLIFERATION

In contrast to PDGF's mitogenic as well as apoptosis-inducing actions, we have identified that LPA acts as a survival (or prevention of apoptosis) factor. Fig 3 contains experimental data that shows the inhibitory action of LPA against PDGF-induced cell death. For this, we stimulated mesangial cells with PDGF, LPA, or both for 2 days, and dead cells were directly counted under fluorescent microscopy after staining the dead nuclei with propidium iodide. As shown in Fig 3, when mesangial cells are stimulated with PDGF, cell death occurs linearly after its addition. According to live/dead cell–number counting, the population undergoing cell death was approximately 5% to 10% of the total stimulated cells. On the other hand, in the presence of LPA, PDGF-induced mesangial cell death is remarkably suppressed. It should be noted that in a quiescent culture condition, mesangial cells exhibit low-grade mesangial cell death, which is also inhibited by LPA. This experiment shows that LPA is able to suppress mesangial cell death triggered by both PDGF or by serum withdrawal. In our additional experiments, we also found that LPA suppressed both DNA fragmentation and the up-expression of p53 evoked by PDGF,[20] given that the LPA's survival-promoting effect was abrogated by LY294002, a selective inhibitor of phosphatidylinositol 3-kinase, one of the most important regulatory proteins involved in antiapoptotic signaling pathways.[23] This suggests that LPA induces mesangial cell survival by inhibiting the apoptotic process of mesangial cells.

Contrasting with earlier-noted antiapoptotic action of LPA, the growth stimulatory action of LPA on its own is extremely minor. Fig 4 shows growth curves of mesangial cells stimulated with PDGF,

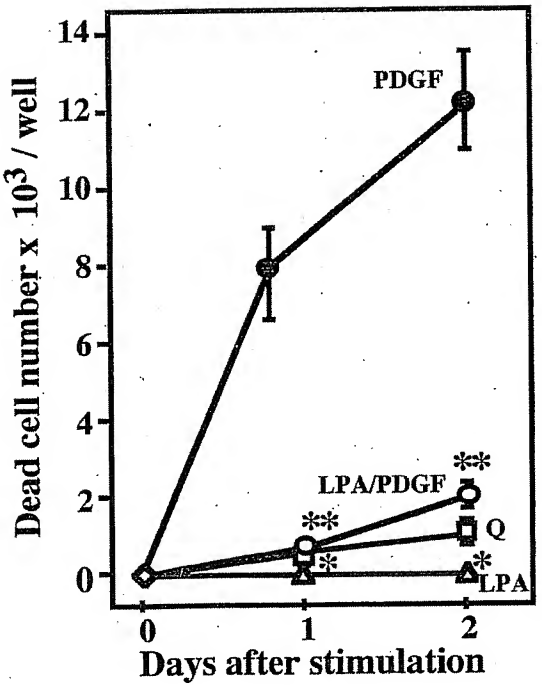

Fig 3. Suppression of mesangial cell death by LPA. Quiescent mesangial cells were stimulated by LPA (30 μmol/L), PDGF (50 ng/mL), or both for 2 days. The whole medium was removed daily and dead cells were separated by gentle centrifugation. Dead cell nuclei were stained with propidium iodide and counted under fluorescent microscopy (n = 3 wells). Cumulative death was plotted against time after treatment (days). LPA suppresses both mesangial cell death evoked by PDGF or by serum withdrawal. *P < .05 versus quiescent cells. **P < .05 versus PDGF-treated cells. Reprinted with permission from Inoue et al: Bimodal effects of platelet-derived growth factor on rat mesangial cell proliferation and death, and the role of lysophosphatidic acid in cell survival. Clin Sci (Colch) 101:11-19, 2001.[20]

LPA, PDGF/LPA, or under quiescent culture conditions. Although PDGF-treated dishes exhibit a 1.6-times higher increase in cell numbers compared with those of quiescent dishes 7 days after the stimulation, the increase in cell numbers in LPA-treated dishes does not significantly differ from those of the quiescent dishes. In contrast, the combination of PDGF and LPA induces synergistically stimulated mesangial cell proliferation with cell numbers reaching 2.5-times that of quiescent dishes at the 7th day. We concluded that LPA is essentially a survival factor for mesangial cells, but behaves as a comitogen when combined with PDGF, enormously exaggerating the mitogenic action of PDGF.

LPA SIGNAL TRANSDUCTION

Our observation that PDGF can stimulate proliferation in cultured mesangial cells without protection from apoptotic cell death, whereas LPA can promote cell survival without proliferation, allows us to separate these 2 events. Together, integrated or coordinated signaling pathways stimulated by both PDGF and LPA are assumed to promote synergistic and enhanced proliferation of mesangial cells. We were interested in how PDGF- and LPA-induced signaling pathways differ, and how both signaling pathways integrated with each other.

LPA is a lipid mediator that acts through G-protein–coupled receptors to evoke various host responses. On the other hand, PDGF interacts with receptor tyrosine kinase, leading to the stimulation of growth responses. First, we focused our attention on the role of the mitogen-activating protein (MAP) kinase in control of the cell survival and proliferation of mesangial cells. MAP kinase is an important cellular signaling component that converts various extracellular signals of growth factors or hormones into intracellular responses through serial phosphorylation cascades and is suggested to play important roles in entering quiescent cells to cell cycle as well as in preventing the cells from apoptosis.[24] In addition, the MAP kinase cascade has been shown to behave as a switch kinase at certain points, capable of integrating signals from tyrosine kinase receptors as well as G-protein–coupled receptors.[25] We measured MAP kinase activity by an immune complex kinase assay using an anti-rat MAP kinase antibody after stimulation of quiescent mesangial cells with LPA, PDGF, or both. We observed that the LPA-induced slow and transient increase in MAP kinase activity peaked at 25 minutes, whereas the PDGF-induced rapid and long activation lasting approximately 50 minutes peaked at 10 minutes. On the other hand, the stimulation of mesangial cells with LPA and PDGF exhibited high and constitutive elevation of MAP kinase activation lasting for more than 50 minutes. PDGF-induced MAP kinase activation was pertussis toxin (PTX)-insensitive, whereas that by LPA was partially (approximately 41%) inhibited by PTX.[26] These results indicate that LPA- and PDGF-induced signaling pathways are distinct, but at the same time they converge at the point of the MAP kinase, inducing high and sus-

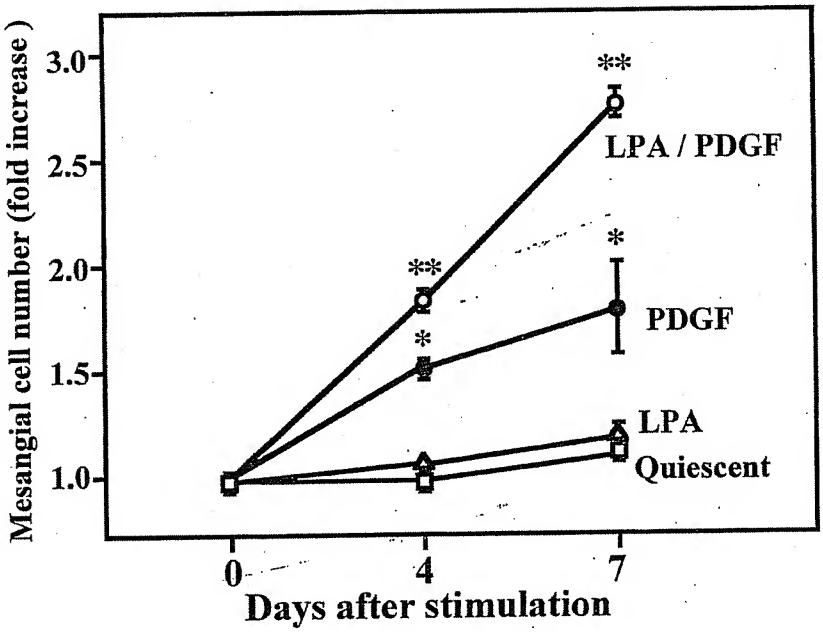

Fig 4. Synergistic growth stimulation by LPA and PDGF. Quiescent mesangial cells were stimulated with LPA (30 μmol/L), PDGF (40 ng/mL), or both for 7 days in RPMI 1640/0.1% bovine serum albumin/0.5% fetal bovine serum. Viable mesangial cells were counted daily by using trypan blue exclusion assay (n = 3 wells). LPA does not stimulate mesangial cell proliferation on its own but synergistically augments the PDGF-induced mesangial cell proliferation. *P < .05 versus quiescent cells. **P < .05 versus PDGF-treated cells. Reprinted with permission from Inoue et al.[37]

tained activation of MAP kinase. When we considered the reports showing that long-lasting and higher magnitude of MAP kinase activation contributes to control both cell proliferation and inhibition from apoptotic cell death in fibroblasts or in Mardin-Darby canine kidney epithelial cells,[24] highly elevated and sustained MAP kinase activation by LPA and PDGF may play a central role in synergistic growth stimulation without cell death in cultured mesangial cells. As for the partial sensitivity to PTX in LPA-induced MAP kinase activation, this may suggest that the PDGF-receptor is trans-activated by the stimulation of LPA receptor as is shown by Goppelt-Struebe et al[27] in mesangial cells.

Among Bcl family proteins, Bcl-xL and Bcl-2 are established survival factors whose physiologic function is to prevent apoptosis. On the other hand, Bax has the opposite effect of Bcl-xL or Bcl-2, accelerating apoptotic cell death.[28] We next investigated the role of such Bcl family proteins in the protective effect of LPA against PDGF-treated cell death. We stimulated mesangial cells with PDGF or LPA, and time-dependent changes in the expression of antiapoptotic Bcl-xL and Bcl-2, and proapoptotic Bax were analyzed by a semiquantitative reverse-transcription polymerase chain reaction method as well as by immunoblot analyses. In our experimental system, exposure of mesangial cells to PDGF did not alter all the messenger RNA (mRNA) and the protein levels of Bcl-xL, Bcl-2, or Bax during the 12 hours after stimulation. On the other hand, LPA significantly up-expressed bcl-xl mRNA and Bcl-xL protein levels 1 and 4 hours after the stimulation, respectively (Fig 5A), whereas LPA did not change the expression of Bcl-2 and Bax. In agreement with this result, antisense oligonucleotide targeted against bcl-xl, which when transfected into the cultured mesangial cells inhibits Bcl-xL expression, induced dose-dependent attenuation of the comitogenic effect of LPA with PDGF (Fig 5B). All of these results suggest that PDGF-stimulated mesangial cells die via apoptosis because of the limitation of the survival factor and that LPA exhibits its survival-promoting action by the induction of antiapoptotic signaling pathways mediated by Bcl-xL.

OTHER MESANGIAL CELLULAR RESPONSES TO LPA

LPA receptors activate multiple secondary messenger pathways involving coupling to G_i, $G_{q/11}$, and $G_{12/13}$. Treatment of mesangial cells with LPA activates PLC and evoked inositol triphosphate (IP_3) formation. The accumulation of IP_3 mobilizes Ca^{2+} from microsome stores, leading to open voltage-gated Ca^{2+} channels.[29] The Ca^{2+} entry into the cells through Ca^{2+} channels promotes mesan-

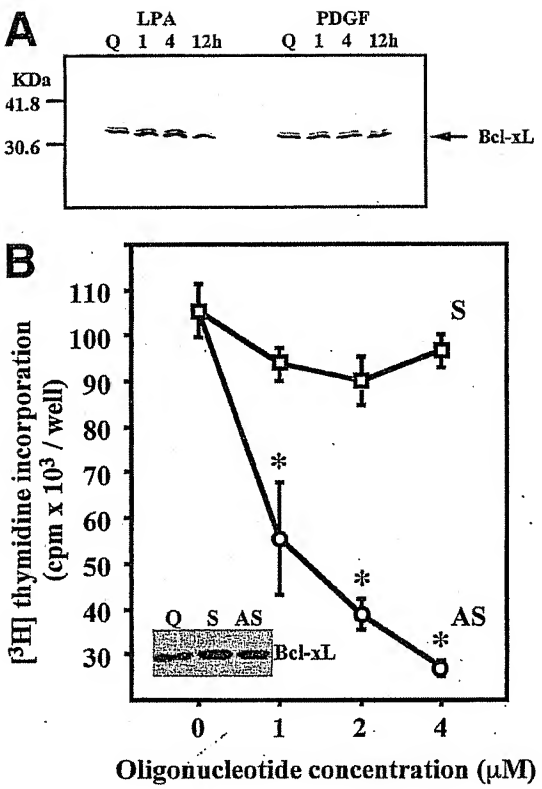

Fig 5. (A) Bcl-xL expression by LPA. Quiescent mesangial cells were stimulated by LPA (30 μmol/L) or PDGF (50 ng/mL) for 1, 4, or 12 hours. Ten micrograms of each cell lysate were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotted with monoclonal anti-Bcl-xL antibody. LPA but not PDGF enhances the expression of Bcl-xL. (B) Inhibition of mesangial DNA synthesis by antisense oligonucleotide against bcl-x. Quiescent rat mesangial cells were pretreated with 0 to 4 μmol/L of antisense (○, AS) or sense (□, S) oligomers[38] for 22 hours and then stimulated with LPA (30 μmol/L)/PDGF(50 ng/mL) for 24 hours. Antisense but not sense oligomer abrogated the comitogenic effect of LPA with PDGF (n = 3 wells). Reduction of Bcl-xL level by antisense oligomer is confirmed. *P < .05 versus sense oligomer-treated cells.

gial cell contraction, similar to various vasoconstrictors including angiotensin II.[30] LPA also stimulates arachidonic acid release and prostaglandin E_2 synthesis through the activation of cytosolic PLA_2.[29] A recent report further shows that LPA exerts Rho-mediated morphologic changes in mesangial cells.[31] Hitherto, details of the classes of G proteins or signaling pathways that lead to these various LPA-induced mesangial responses remained to be fully understood. In our observation, IP_3-dependent Ca^{2+} mobilization is completely PTX sensitive, suggesting that LPA-induced activation of G_i may link to PLC in mesangial cells, and that this is different from that in cultured fibroblasts, where the PTX-insensitive $G_{q/11}$ pathway activates PLC.[32] Obviously, signaling pathways and networks downstream of LPA receptors appear to diverge depending on LPA receptor subtypes or the cell types.

LPA AS A DETERMINANT OF MESANGIAL GROWTH

We now speculate, as in Fig 6, as to the molecular basis of the synergistic growth stimulation of mesangial cells by LPA and PDGF. Among growth factors that evoke mesangial cell proliferation, PDGF behaves as a principal element, acting not only directly but also as an intermediary for other growth factors, including endothelin-1 and TNF-α.[18,33] Stimulation of mesangial cells with PDGF induces various growth-related oncogene expressions such as c-myc and also activates MAP kinase, leading to induction mitogenic responses.[26,34] However, because of the inability of PDGF to induce survival signals such as Bcl family genes, the cycling cells do not successfully complete the cell cycle, leading to abortive proliferation, in which continuous mesangial cell death occurs side by side with mesangial cell proliferation. A similar type of limited cell proliferation has been observed in a hybridoma cell line.[35] This phenomenon may be interpreted as a beneficial mechanisms whereby the hyperplasia of mesangial cells can be counterbalanced by the accompanied death in mesangial cells when just PDGF is involved in the proliferation of mesangial cells in vivo in diseased glomeruli.

On the other hand, when LPA co-acts with PDGF, Bcl-xL is up-expressed and provides signals that suppress mesangial apoptotic response. Under the supplementation of this survival factor, mesangial cells avoid the death response and continue to proliferate, leading to synergistically augmented mesangial cell growth. Because LPA is generated by the action of group II PLA_2 when plasma membranes are injured or during the clotting of platelets, such uncontrolled mesangial growth would occur when mesangial and/or vascular endothelial cell membranes are damaged or blood coagulability is elevated within inflamed glomeruli. Furthermore, LPA can stimulate arachidonic acid production as well as induce platelet

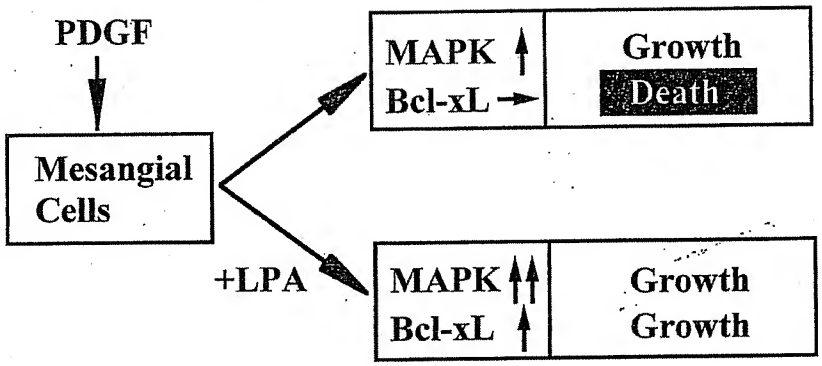

Fig 6. Hypothetical mechanism of abortive proliferation by PDGF and sustained proliferation by LPA and PDGF. Stimulation of mesangial cells with PDGF induces activation of MAP kinase (MAPK), leading to induction of mitogenic responses. However, because of the inability of PDGF to induce survival signals, the cycling cells do not successfully complete the cell cycle, leading to abortive proliferation, in which continuous cell death occurs side by side with cell proliferation. On the other hand, when LPA co-acts with PDGF, antiapoptotic Bcl-xL is up-expressed and inhibits mesangial apoptotic response, leading to synergistically augmented mesangial cell proliferation. We suggest that this mechanism may be involved in the process of protractedly sustained proliferative glomerulonephritis.

aggregation, suggesting that LPA autocrinely or paracrinely augments or exacerbates the pathologic process of mesangial cell proliferation and inflammation. Indeed, a low incidence of apoptotic mesangial cell death has been shown to correlate with a clinically protracted resolution of renal diseases in lupus nephritis and immunoglobulin A nephropathy.[36] We have found Edg-4 receptor mRNA to be highly expressed in renal glomeruli isolated from biopsy samples of patients with advanced mesangial proliferative glomerulonephritis of immunoglobulin A nephropathy. We have also recently determined that both Edg-2 and Edg-4 mRNAs are abundantly expressed in primary cultured human mesangial cells (C. N. Inoue, unpublished work). This seems to be evidence that LPA is generated in diseased glomeruli and that it is indeed involved in the proliferation of mesangial cells.

Here, we suggest that inappropriate equilibrium of mesangial cell growth and death resulting from improper induction of antiapoptotic LPA may be involved in the etiology of proliferative glomerulonephritis. Although the details of the downstream signaling pathways of LPA remain to be fully elaborated, we believe that intervention of the proliferative consequence of LPA would become an important strategy for the therapy of progressively protracted mesangial proliferative glomerulonephritis.

REFERENCES

1. Schumacher KA, Classen HG, Spath M: Platelet aggregation evoked in vitro and in vivo by phosphatidic acids and lysoderivatives: Identity with substances in aged serum (DAS). Thromb Haemost 42:631-640, 1979

2. Moolenaar WH: Development of our current understanding of bioactive lysophospholipids. Ann N Y Acad Sci 905:1-10, 2000

3. An S, Dickens MA, Bleu T, et al: Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. Biochem Biophys Res Commun 231:619-622, 1997

4. Goetzl EJ, Lee H, Azuma T, et al: Gelsolin binding and cellular presentation of lysophosphatidic acid. J Biol Chem 275:14573-14578, 2000

5. Goetzl EJ: Pleiotypic mechanisms of cellular responses to biologically active lysophospholipids. Prostaglandins 64:11-20, 2001

6. Eichholtz T, Jalink K, Fahrenfort I, et al: The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem J 291:677-680, 1993

7. le Balle F, Simon MF, Meijer S, et al: Membrane sidedness of biosynthetic pathways involved in the production of lysophosphatidic acid. Adv Enzyme Regul 39:275-284, 1999

8. Fourcade O, Simon MF, Viode C, et al: Secretory phospholipase A_2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. Cell 80:919-927, 1995

9. van Dijk MC, Postma F, Hilkmann H, et al: Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca^{2+} signaling pathways. Curr Biol 8:386-392, 1998

10. Dorsam G, Taher MM, Valerie KC, et al: Diphenyleneiodium chloride blocks inflammatory cytokine-induced up-regulation of group IIA phospholipase A(2) in rat mesangial cells. J Pharmacol Exp Ther 292:271-279, 2000

11. Wada A, Tojo H, Sugiura T, et al: Group II phospholipase A_2 as an autocrine growth factor mediating interleukin-1 action on mesangial cells. Biochim Biophys Acta 1345:99-108, 1997

12. Liliom K, Guan Z, Tseng JL, et al: Growth factor-like phospholipids generated after corneal injury. Am J Physiol 274:C1065-C1074, 1998

13. Siess W, Zangl KJ, Essler M, et al: Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc Natl Acad Sci U S A 96:6931-6936, 1999

14. Lee HS: Oxidized LDL, glomerular mesangial cells and collagen. Diabetes Res Clin Pract 45:117-122, 1999

15. An S: Molecular identification and characterization of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. Ann N Y Acad Sci 905:25-33, 2000

16. Kreisberg JI, Karnovsky MJ: Glomerular cells in culture. Kidney Int 23:439-447, 1983

17. Floege J, Radeke HR, Johnson RJ: Glomerular cells in vitro versus the glomerulus in vivo. Kidney Int 45:360-368, 1994

18. Silver BJ, Jaffer FE, Abboud HE: Platelet-derived growth factor synthesis in mesangial cells: Induction by multiple peptide mitogens. Proc Natl Acad Sci U S A 86:1056-1060, 1989

19. Abboud HE: Platelet-derived growth factor and mesangial cells. Kidney Int 41:581-583, 1992

20. Inoue CN, Nagano I, Ichinohasama R, et al: Bimodal effects of platelet-derived growth factor on rat mesangial cell proliferation and death, and the role of lysophosphatidic acid in cell survival. Clin Sci (Colch) 101:11-19, 2001

21. Wyllie AH: Apoptosis: An overview. Br Med Bull 53: 451-465, 1997

22. Amundson SA, Myers TG, Fornace AJ Jr: Roles for p53 in growth arrest and apoptosis: Putting on the brakes after genotoxic stress. Oncogene 17:3287-3299, 1998

23. Carpenter CL, Cantley LC: Phosphoinositide kinases. Curr Opin Cell Biol 8:153-158, 1996

24. Le Gall M, Chambard JC, Breittmayer JP, et al: The p42/p44 MAP kinase pathway prevents apoptosis induced by anchorage and serum removal. Mol Biol Cell 11:1103-1112, 2000

25. Malarkey K, Belham CM, Paul A, et al: The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. Biochem J 309:361-375, 1995

26. Inoue CN, Ko YH, Guggino WB, et al: Lysophosphatidic acid and platelet-derived growth factor synergistically stimulate growth of cultured rat mesangial cells. Proc Soc Exp Biol Med 216:370-379, 1997

27. Goppelt-Struebe M, Fickel S, Reiser CO: The platelet-derived-growth-factor receptor, not the epidermal-growth-factor receptor, is used by lysophosphatidic acid to activate p42/44 mitogen-activated protein kinase and to induce prostaglandin G/H synthase-2 in mesangial cells. Biochem J 345:217-224, 2000

28. Korsmeyer SJ: BCL-2 gene family and the regulation of programmed cell death. Cancer Res 59:1693S-1700S, 1999 (suppl 7)

29. Inoue CN, Forster HG, Epstein M: Effects of lysophosphatidic acid, a novel lipid mediator, on cytosolic Ca^{2+} and contractility in cultured rat mesangial cells. Circ Res 77:888-896, 1995

30. Takeda K, Meyer-Lehnert H, Kim JK, et al: Effect of angiotensin II on Ca^{2+} kinetics and contraction in cultured rat glomerular mesangial cells. Am J Physiol 254:F254-F266, 1988

31. Reiser CO, Lanz T, Hofmann F, et al: Lysophosphatidic acid-mediated signal-transduction pathways involved in the induction of the early-response genes prostaglandin G/H synthase-2 and Egr-1: A critical role for the mitogen-activated protein kinase p38 and for Rho proteins. Biochem J 330:1107-1114, 1998

32. An S, Goetzl EJ, Lee H: Signaling mechanisms and molecular characteristics of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. J Cell Biochem Suppl 30-31:147-157, 1998

33. Gesualdo L, Di Paolo S, Ranieri E, et al: Growth factors and their receptors in the progression of renal damage. Contrib Nephrol 111:38-44, 1995

34. Makino T, Ono T, Muso E, et al: Inhibitory effects of rosmarinic acid on the proliferation of cultured murine mesangial cells. Nephrol Dial Transplant 15:1140-1145, 2000

35. Chung JD, Sinskey AJ, Stephanopoulos G: Growth factor and bcl-2 mediated survival during abortive proliferation of hybridoma cell line. Biotechnol Bioeng 57:164-171, 1998

36. Soto H, Mosquera J, Rodriguez-Iturbe B, et al: Apoptosis in proliferative glomerulonephritis: Decreased apoptosis expression in lupus nephritis. Nephrol Dial Transplant 12:273-280, 1997

37. Inoue CN, Epstein M, Forster HG, et al: Lysophosphatidic acid and mesangial cells: Implications for renal diseases. Clin Sci (Colch) 96:431-436, 1999

38. Fujio Y, Kunisada K, Hirota H, et al: Signals through gp130 upregulate bcl-x gene expression via STAT1-binding cis-element in cardiac myocytes. J Clin Invest 99:2898-2905, 1997

Acta Diabetol 29: 142–148, 1992

ACTA
DIABETOLOGICA

Diabetic nephropathy in type 2 diabetes mellitus: animal models with emphasis on recently developed corpulent rat strains

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Abstract. Genetic background has recently been implicated as an important factor in the development of diabetic nephropathy in humans. Little is known about the natural history of renal disease in patients with type 2 (non-insulin-dependent) diabetes mellitus. Because toxins used in chemically induced models of diabetes may be associated with pathological renal changes, investigators have employed well-defined, genetically distinct animal models to study diabetic nephropathy. This review will focus on animal models of spontaneous type 2 diabetic nephropathy. No single animal model of diabetes corresponds exactly to the human disorder, nor develops renal changes identical to those seen in man. Rodents have been the most studied species with diabetic renal disease. The spontaneously hypertensive/NIH-corpulent (SHR/N-*cp*) rat provides a unique model of obesity, type 2 diabetes and nephropathy. In addition, genetic controls for this rat, including the lean SHR/N-*cp* rat and the Wistar-Kyoto/NIH-corpulent (WKY/N-*cp*) rat, allow assessment of the role of hypertension and obesity in the pathogenesis of diabetic nephropathy. SHR/N-*cp* rats have abnormal glucose tolerance, hypertension, and develop a renal disease reminiscent of human diabetic nephropathy. WKY/N-*cp* rats are also obese and have hyperlipidaemia, but their glucose control is somewhat worse than that of the SHR/N-*cp* rat. In contrast, they do not have hypertension, the renal disease is less severe, and is less suggestive of the human pathology. Finally the LA/N-*cp* rat also carries the gene for obesity, and exhibits hyperlipidaemia. In contrast to the other two spontaneous models of obesity it does not exhibit hypertension or glucose intolerance. There is little evidence of renal disease in this model. The development of genetic models of obesity, hypertension and glucose intolerance provides a unique opportunity to make longitudinal observations on the natural history of diabetic nephropathy, and to test the various proposed mechanisms for the development of diabetic glomerulopathy, and the effects of various treatment modalities. Diversity in manifestations of diabetic renal disease in animals suggests the importance of genetic mechanisms in pathological outcomes.

Key words: Hyperlipidaemia – Hypertension – Mice – Obesity – Rats

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Introduction

The pathogenesis of diabetic nephropathy is not completely understood. Clinical studies are limited because renal disease usually develops insidiously over several years in the diabetic patient before the clinical manifestations of nephropathy are evident. Genetic factors have been implicated as playing a major role in the development of diabetic nephropathy in humans [1, 2]. Genetic predisposition to glomerular injury could explain why only as few as one third of patients with type 1 (insulin-dependent) diabetes mellitus are susceptible to diabetic nephropathy. Much less is known about the natural history and course of renal disease in patients with type 2 (non-insulin-dependent) diabetes mellitus [3, 4]. The role of insulin resistance in the pathogenesis of hypertension or its direct effect on renal function is currently being studied. However, genetic differences may encompass such factors as obesity, hypertension, and metabolic and hormonal responses, which might modulate the expression of diabetic nephropathy within and between animal strains. Toxin-induced models of diabetes may be associated with nephrotoxic pathological changes. Investigators have therefore employed well-defined, genetically distinct animal models of diabetes mellitus to make direct observations on the natural history of the renal disease and the progression of renal structural and functional changes.

There are currently a variety of spontaneously hyperglycaemic animals used as models for human diabetes [5]. In this review we describe animal models of spontaneous type 2 diabetic nephropathy.

Animal models of spontaneous diabetes

A number of animal species exist which spontaneously develop diabetes mellitus. Diabetes in these animals is manifested by a variety of clinical symptoms similar to those of the different forms of human diabetes. As in humans, conditions of hypoinsulinaemia analogous to type 1 diabetes and hyperinsulinaemia resembling type 2 diabetes are also observed in animals. The expression and severity of metabolic, hormonal and pathological abnormalities may vary according to the genetic background, nutrition, age and species. Because of this heterogeneity no single animal model corresponds exactly to the human disorder. Similarly, while diabetic animals may develop kidney disease which resembles human diabetic nephropathy, no diabetic animal develops renal changes identical to those seen in man (Table 1). We will concentrate in this review on models of type 2 diabetes mellitus characterized by hyperglycaemia and hyperinsulinaemia.

Spontaneously diabetic animals with kidney complications

Primates

Macaca mulatta. Recently, renal pathology has been examined in obese rhesus monkeys with type 2 diabetes [6]. Monkeys with established diabetes have proteinuria and azotaemia. Kidneys exhibit diffuse glomerulosclerosis, tubular atrophy and interstitial fibrosis. Nodular lesions were observed [7]. Animals with normal glucose metabolism have normal renal histology.

Table 1. Renal lesions in humans and animals with spontaneous type 2 diabetes mellitus

Type of lesions	RE	DME	ND	GBMT	EH	AH
Humans	+	+	+	+	+	+
Monkey						
Obese rhesus (*Macaca mulatta*)		+	+			
Rats						
Cohen	+	+		+	+	
Obese SHR (Koletsky)	+	+		+		
SHR/N-*cp*	+	+	+		+	+
SHHF/Mcc-*cp*	+	+	+			
WKY/N-*cp*	+	+				
WDF/Ta-*fa* (Wistar fatty)	+	+		+		
Mice						
ob/ob		+				
db/db	+	+		+	+	
KK	+	+	+	+	+	
KKAy				+		
NZO	+	+	+	+		+
Desert rodents						
Sand rat		+				
Spiny mouse				+		
Tuco-tuco		+		+		+

RE, renal enlargement; DME, diffuse mesangial expansion; ND, nodular lesions; GBMT, glomerular basement membrane thickening; EH, exudative hyaline lesions; AH, arteriolar hyalinosis; +, reported

Rats

Cohen diabetic rat. This strain was developed by genetic selection of albino rats from the Hebrew University strain for their propensity to develop overt diabetes and diabetes-related complications when fed a diet containing a high percentage of refined sugar [8]. Diabetes in Cohen rats is characterized by hyperglycaemia, glucosuria and hyperinsulinaemia, with late development of hypoinsulinaemia, insulin resistance, and decreased number and sensitivity of insulin receptors. When Cohen rats are fed a starch or stock diet no overt diabetes or nephropathy develops.

Nephropathy is common and occurs in about 50%–60% of diabetic Cohen rats [9]. Proteinuria and renal lesions are noted as early as 4 months of age. The kidneys are grossly enlarged. Renal lesions consist of diffuse glomerulosclerosis with acellular thickening of the mesangium and of the peripheral glomerular basement membrane (GBM), and segmental lipohyaline-exudative changes resembling the human "hyalin cap" lesion. Glomerular xanthomatous and microaneurysm-like lesions have also been described. Arterial and arteriolar sclerosis, and tubular atrophy and cystic dilatation may be seen.

Obese spontaneously hypertensive (obese SHR) rat. This rat strain was developed by Koletsky by mating a female SHR of the Kyoto-Wistar strain with a normotensive Sprague-Dawley male [10]. After several generations of selective inbreeding, the obese SHR spontaneously appeared and exhibited obesity, hypertension and hyperlipidaemia. In addition, some obese SHRs developed hyperglycaemia and glucosuria associated with giant hyperplasia of pancreatic islets. Hypertension occurs early and precedes the development of kidney disease.

Renal disease in obese SHR rats is manifested at a very early age by proteinuria followed by progressive glomerular, vascular and tubular damage and subsequent azotaemia and uraemia [11]. Glomerular lesions consist of mesangial expansion and hypercellularity, along with focal necrosis, collagen deposition and hyalinization. Thickening of the capillary basement membrane has been observed. Arteriolar nephrosclerosis is also prominent. Glomerular and vascular lesions progress leading to glomerulosclerosis, interstitial fibrosis and tubular atrophy. Most animals die from uraemia or complications of vascular disease.

Wistar diabetic/Ta-fatty rat (WDF/Ta-fa) rat. This strain, commonly referred to as the "Wistar fatty" rat, is a genetically obese, hyperglycaemic rat established by transfer of the fatty (*fa*) gene from the Zucker rat (13 M strain) to the Wistar-Kyoto rat [12]. After five generations of backcrosses, the homozygous Wistar fatty (*fa/fa*) rat exhibits obesity, hyperinsulinaemia, glucose intoler-

ance, hyperlipidaemia, and hyperphagia similar to Zucker rats. However, Wistar fatty rats are more glucose intolerant and insulin resistant than Zucker rats. Diabetic symptoms, such as hyperglycaemia, polydipsia and glucosuria, appear as early as 8 weeks in male rats and worsen with advancing age. Hyperglycaemia is usually not observed in females but can be induced by addition of sucrose to the diet [13].

Renal disease in Wistar fatty rats is marked by onset of albuminuria and decreased glomerular filtration rate at 20 weeks of age [14]. Progressive increases in albuminuria occur until 7 months of age. At this time albumin excretion rates may be 25 times those of lean controls. Kidney enlargement and glomerular hypertrophy are observed at 20 and 42 weeks. Glomerular and mesangial volume and GBM surface area and thickness are significantly increased in 42-week-old Wistar fatty rats compared with lean controls.

Spontaneously hypertensive/NIH-corpulent (SHR/N-cp) rat. This congenic rat strain, developed by C. T. Hansen at the National Institutes of Health (NIH), was derived by mating a male Koletsky rat heterozygous for the corpulent gene (*cp*/+) to a female SHR rat of the Okamoto strain [15]. After a minimum of 12 backcrosses (fully congenic), homozygous (*cp*/*cp*) SHR/N-*cp* rats exhibit obesity, mild hypertension, hyperlipidaemia, hyperinsulinaemia and glucose intolerance that resembles type 2 diabetes mellitus [16] (Table 2). Similarly to the *fa*/*fa* rat, expression of diabetes in the *cp*/*cp* rat depends on the genetic background on which it is maintained [17]. Males exhibit greater glucose intolerance than females and develop glucosuria [18].

Obese SHR/N-*cp* rats develop significant proteinuria and nephropathy as early as 3 months after being fed high-carbohydrate diets [19]. The kidneys are enlarged with hypertrophied glomeruli [20]. Diffuse and segmental mesangial expansion and an increased number of mesangial cells are the predominant glomerular lesions. In some glomeruli, nodular lesions and intracapillary exudative lesions are occasionally observed. Focal segmental glomerulosclerosis (FGS), a lesion normally seen in aged rats, is also present in some glomeruli. Thickening of peripheral GBM and arteriolar hyalinization have been rarely observed. Both the diabetic state and the proteinuria and glomerular lesions are accentuated by feeding a high-sucrose diet [19, 21].

Spontaneously hypertensive heart failure/McCune-corpulent (SHHF/Mcc-cp) rat. The SHHF/Mcc-*cp* rat is another strain developed from backcrossing the *cp* mutation onto the SHR/N background. After 7 backcrosses rats were sent from the National Institutes of Health to S. McCune (Ohio State University), and have subsequently been inbred for the occurrence of heart failure [22]. Obese rats (*cp*/*cp*) exhibit hyperglycaemia, hyperinsulinaemia, hypertriglyceridaemia and mild hypercholesterolaemia [23]. Hypertension is more severe in obese SHHF/Mcc-*cp* than in obese SHR/N-*cp* rats. Obese SHHF/Mcc-*cp* rats also demonstrate fasting hyperglycaemia and develop myocardial disease not present in obese SHR/N-*cp* rats. The expression of diabetes and diabetic complications is less severe in obese female than obese male SHHF/Mcc-*cp* rats.

Obese SHHF/Mcc-*cp* rats exhibit progressive proteinuria as early as 4 months of age and subsequently develop loss of renal function [24]. Renal function is more severely altered in rats with heart failure. Glomerular changes reported consist of increased cellularity with diffuse mesangial expansion. Mesangial nodules are rarely observed.

Wistar-Kyoto/NIH-corpulent (WKY/N-cp) rat. The congenic strain WKY/N-*cp* was recently developed by C. T. Hansen at NIH and was metabolically characterized by O. E. Michaelis at the Beltsville Human Nutrition Research Center. The strain was derived by introducing the *cp* gene into the WKY/N background from the SHR/N-*cp* strain, followed by 12 backcrosses [22]. As with obese male SHR/N-*cp* rats [25], obese male WKY/N-*cp* rats (*cp*/*cp*) exhibit hyperglycaemia, hyperinsulinaemia, hyperlipidaemia and glucosuria (Table 2). However, in contrast to obese SHR/N-*cp* rats [25], obese WKY/N-*cp* rats are normotensive [systolic blood pressure 115±3 mmHg by tail cuff (for $n=12$)], and exhibit fasting hyper-

Table 2. Parameters of glucose tolerance, serum lipid levels and indices of renal function in obese and lean male WKY/N-*cp* and SHR/N-*cp* rats

	WKY/N-*cp*		SHR/N-*cp*	
	Obese	Lean	Obese	Lean
Body weight (g)	622±23	413±51[a]	594±12	423±7[a]
Serum glucose (mg/dl)				
Fasting	207±24[b]	123±6[a]	124±6	109±3[a]
1-h response	415±35	169±9[a]	351±19	150±6[a]
Serum insulin (μU/ml)				
Fasting	285±30[b]	97±6[a]	486±34	74±6[a]
1-h response	401±31[b]	134±5[a]	881±99	129±12[a]
Serum triglyceride (mg/dl)				
Fasting	286±14[b]	103±6[a]	412±47	94±7[a]
Serum total cholesterol (mg/dl)				
Fasting	218±15	88±3[a]	223±14	96±3[a]
Urinary glucose (mg/17 h)	164±58	1.6±0.2[a]	191±37	1.0±0.1[a]
Urinary total protein (mg/17 h)	46±3[b]	33±3[a]	87±9	34±2[a]
Creatinine clearance (ml/min per kg)	1.54±0.35	2.85±0.34	2.14±0.47	3.52±0.36

Young rats (5–6 weeks old) were fed a nutritionally adequate semipurified diet containing 54% carbohydrate for 3 months [43]. Blood was obtained by tail bleeding. Fasting serum parameters were determined in the early portion of the dark cycle after rats were fasted for 12–14 h. Glucose tolerances and insulin responses were determined in fasted rats following an oral glucose load of 250 mg/100 g body weight. Urine was collected from non-fasted rats. After 6 h in the dark cycle rats were put into metabolic cages without food for 17 h. Results are means ± SEM for at least 12 animals

[a] $P<0.05$, lean different from obese within parameter and strain

[b] $P<0.05$, obese WKY/N-*cp* different from obese SHR/N-*cp* within parameter and phenotype

glycaemia. Levels of serum insulin and triglycerides are higher in obese SHR/N-*cp* than WKY/N-*cp* rats, perhaps reflecting strain differences in pancreatic insulin secretion in response to glucose [26].

Obese WKY/N-*cp* rats develop enlarged kidneys, proteinuria and glomerulopathy associated with type 2 diabetes mellitus by 3 months after being fed high-carbohydrate diets (Table 3). Glomerular changes observed were hypercellularity associated with segmental and diffuse mesangial expansion. Nephropathy is less severe in obese WKY/N-*cp* rats than previously reported for obese SHR/N-*cp* rats [19]. Such differences might be explained by differences in systemic blood pressure.

Mice

Obese mouse (ob/ob). The obese mouse was discovered as a spontaneous autosomal recessive mutation, presenting with massive obesity and marked hyperglycaemia [27]. The *ob* mutation was transferred later and maintained in different inbred strains. Depending on the inbred background, the *ob/ob* mouse may express different obesity-diabetic syndromes. The *ob* mutant maintained on the C57BL/6J background (C57BL/6J *ob/ob*) develops massive obesity, mild glucose intolerance, transient hyperglycaemia and severe hyperinsulinaemia associated with hyperplastic pancreatic islets. In contrast, the C57BL/KsJ *ob/ob* mouse develops severe diabetes marked by initial transient hyperinsulinaemia followed by rapidly developing insulinopenia, beta-cell atrophy and severe hyperglycaemia, leading to early death.

Renal lesions are not generally seen in *ob/ob* mice. However, diffuse and nodular lipohyaline changes in glomeruli and deposition of hyaline material within capillary walls have been reported in some animals [28].

Diabetes mouse (db/db). The diabetic mouse is another strain derived from an autosomal recessive mutation (db) [29]. This mutation occurred spontaneously in mice of C57BL/KsJ background. On this background the diabetic obese mouse (C57BL/KsJ *db/db*) consistently develops a severe diabetic syndrome similar to that in the C57BL/KsJ *ob/ob* mouse, characterized by early onset of hyperinsulinaemia followed by increasing hyperglycaemia with hypoinsulinaemia, weight loss and early death. Diabetes is more severe with this mutation compared with other obese mutations. The *db* gene has also been introduced into the C57BL/6J background. The C57BL/6J *db/db* mouse, like the *ob/ob* mouse with the same genetic background, develops obesity with mild hyperglycaemia, and hyperinsulinaemia. The *db/db* mouse, in contrast to the *ob/ob* mouse, develops significant nephropathy [30, 31]. Renal disease is marked by increased glomerular filtration rate at onset of diabetes, followed by the appearance of proteinuria. As the disease advances glomerular filtration rate decreases in older mice. Structurally, the kidneys are enlarged and show diabetic lesions including diffuse and nodular thickening of mesangial matrix, increased number of mesangial cells, hyaline exudative lesions, deposition of collagen fibrils and vacuolar changes in glomeruli, and characteristic nodular GBM thickening. Deposition of immune complexes in mesangium and tubules has also been observed.

Table 3. Kidney weight and glomerulopathy in obese and lean male WKY/N-*cp* rats

	Obese	Lean
Kidney weight (g)	1.65±0.05[a]	1.28±0.02
Glomeruli (%)		
Normal	67±5[a]	97±1
Segmental mesangial expansion	28±3[a]	3±1
Diffuse mesangial expansion	5±2[a]	0

Young rats (5–6 weeks old) were fed a nutritionally adequate semipurified diet containing 54% carbohydrate for 3 months [43]. Histological preparation and examination was conducted by A. Abraham, Department of Pathology, George Washington University Medical Center, Washington, DC. Kidneys were fixed in buffered 10% formalin and embedded in paraffin. Sections cut at 3 μm for light microscopy were stained with haematoxylin and eosin. One hundred glomeruli from each kidney were counted and represented most of the glomeruli in a coronal section of a rat kidney. Results are means ±SEM for at least 6 animals.

[a] $P<0.05$, obese different from lean

KK mouse. The KK mouse is one of a series of genetically diabetic strains developed originally by Kondo [32]. The mode of inheritance is not understood but is assumed to be polygenic. This strain is characterized by slowly developing obesity, mild hyperglycaemia with hyperinsulinaemia, and associated increases in the number and size of pancreatic islets. Metabolic abnormalities are maximal at 4–9 months and normalize by 1 year. Because of polygenic inheritance there is a lack of an appropriate control mouse with which to make comparisons.

Renal lesions in KK mice closely resemble those in human diabetic nephropathy [33, 34]. Glomeruli of diabetic KK mice show diffuse and nodular enlargement of the mesangium with proliferation of mesangial cells. However, the nodular lesions are not identical to the Kimmelstiel-Wilson nodules seen in human diabetic nephropathy. Nodular lesions in KK mice are usually located near the hilus and are less prominent and extensive than human Kimmelstiel-Wilson lesions. Immunohistological studies show an intense, specific fluorescence for gamma globulin in the enlarged mesangium, in nodules and along the capillary wall. Exudative "fibrinoid caps" are also observed in glomeruli of 13- to 16-month-old animals, but are rarely seen in very young or older animals. Membranous changes and splitting of the basement membrane due to deposition of newly formed basement membrane material are observed in some animals. Proteinuria in diabetic mice increases with advancing age. The extent of mesangial enlargement correlates with the degree of hyperglycaemia.

KKAy mouse. This strain was originally bred from mating black KK females to yellow KKAy males at the Takeda Chemical Corporation, Osaka, Japan [35]. It differs from the KK mouse in that it carries the yellow obese and diabetic genes, whereas the KK mouse carries only the

diabetic gene. KKAy mice are obese and have early-onset and prolonged severe hyperinsulinaemia and hyperglycaemia.

Renal involvement is uniquely marked by the early onset and rapid development of GBM thickening which is evident by 3 months and tends to increase with age [35], in contrast to the slow GBM thickening seen in other diabetic animal models. The GBMs of KKAy mice show a predominantly smooth contour with infrequent nodules.

New Zealand obese (NZO) mouse. This strain was developed by selective inbreeding of obese mice from a mixed colony, beginning with a pair of agouti mice, which has also given rise to the NZB (black) strain [36]. NZO mice have a high prevalence of autoimmune disorders. They develop obesity, mild hyperglycaemia, glucose intolerance, hyperinsulinaemia and insulin resistance. Hyperglycaemia and glucose intolerance increase continuously with advancing age of the animals.

Renal disease in NZO mice is seen by 6 months of age [36]. Kidneys show various lesions: mild to severe glomerular proliferation, mesangial deposits, glomerulosclerosis, mild GBM thickening, occasioal hyalinization of glomerular arterioles, and arteriolar inflammation. Eosinophilic nodules are seen in some glomeruli. Histological changes are associated with granular deposition of immunoglobulins in glomeruli similar to those seen in glomeruli of diabetic KK mice; however, their pathogenetic significance is unclear.

Desert rodents

The sand rat (*Psammomys obesus*), spiny mouse (*Acomys cahirinus*) and tuco-tuco (*Ctenomys talarum*) are three species of rodent whose natural habitat is the arid areas of the world (respectively, the Middle East, Eastern Mediterranean and Argentina [37]). When these rodents are transferred into the laboratory with a readily available supply of high-calorie chow they develop a syndrome resembling type 2 diabetes mellitus, namely, hyperphagia, obesity, hyperglycaemia, hyperinsulinaemia and cataracts.

Sand rat (Psammomys obesus). The diabetic syndrome in the sand rat usually develops within 2–3 months [38]. Severity of diabetes varies between individual animals. Severely hyperglycaemic animals die prematurely from ketosis. Initially, the pancreatic islets appear normal, but later degranulation of pancreatic beta-cells is observed. This is followed by beta-cell degeneration and necrosis with resultant insulinopenia and ketonuria.

The kidneys of sand rats are usually normal. In severely diabetic animals glycogen-containing tubular vacuoles are frequently seen. In these animals no glomerular changes or GBM abnormalities have been found [39].

Spiny mouse (Acomys cahirinus). Variations in the appearance and severity of diabetes and obesity also occur in this species [40]. Some animals show obesity, mild hyperglycaemia and hyperinsulinaemia. Regardless of the stage of the disease, spiny mice characteristically have massive pancreatic islet hyperplasia and increased pancreatic insulin content. Despite the large insulin stores, plasma insulin response to glucose is delayed or impaired, suggesting an impairment of the hormone's release mechanism.

Renal abnormalities in spiny mice consist of increased mesangial and collagen fibres within the mesangium and nodular thickening of the glomerular GBM [41]. Glycogen deposition in tubular cells is also observed.

Tuco-tuco (Ctenomis talarus). The diabetic syndrome in the tuco-tuco is similar to that in the sand rat and spiny mouse [42]. However, the tuco-tuco tends to be less prone to hyperglycaemia and ketosis. Many animals, especially males, become hyperphagic and very obese. Degranulation of beta-cells is the usual lesion in the pancreas but amyloid hyalinization of islets has been observed in a few animals. Glomerular lesions have been reported in the tuco-tuco [42]. Diffuse mesangial argyrophilia and GBM thickening in peripheral capillary loops were noted in most animals, but nodular glomerulosclerosis or tubular lesions were not present. Hyaline thickening of afferent arterioles was evident in some animals.

Summary

Kidney disease appears to be a common complication of spontaneous diabetes mellitus in many animal species. It occurs in animals with either hypoinsulinaemia or hyperinsulinaemia. The renal functional and structural abnormalities in these animals resemble human diabetic nephropathy in many respects. GFR was increased in some diabetic animals in which it was studied. Diffuse expansion of the mesangial matrix (diffuse glomerulosclerosis) and GBM thickening, two structural hallmarks of diabetic glomerulopathy in humans, are the most frequently encountered lesions in animals. The majority of models such as obese rhesus monkeys, Cohen rats, obese SHR/N-*cp*, SHHF/Mcc-*cp* and WKY/N-*cp* rats, and *db*/*db*, KK and NZO mice, and others, exhibit mesangial expansion; whereas others, like the BB rat, and NOD and KKAy mice, show only GBM widening. In addition to diffuse glomerulosclerosis, a nodular form of mesangial enlargement, resembling (but not identical with) human nodular glomerulosclerosis or Kimmelstiel-Wilson lesions, has been observed in obese rhesus monkeys, obese SHR/N-*cp* and SHHF/Mcc-*cp* rats and NZO and KK mice. Exudative lesions resembling human hyaline or fibrin caps have also been found in the diabetic Cohen and SHR/N-*cp* rat, and *db*/*db* and KK mice. Renal arteriolar hyalinosis, another characteristic lesion frequently seen in humans, is not present in most animal models but has been observed occasionally in the SHR/N-*cp* rat, NZO mouse and the desert rodent, tuco-tuco. Although the glomerular lesions observed in spontaneously diabetic animals do not conform exactly with human diabetic glomerulopathy, the dominant change in glomerular

structure appears to be the same as in humans, characterized by excessive accumulation of mesangial matrix and GBM material.

An obvious common link to the diabetic renal lesions has been the presence of hyperglycaemia. However, several possible mechanisms, including metabolic, humoral, haemodynamic and genetic factors, which may contribute to the initiation and progression of diabetic renal changes have been investigated in patients with type 1 diabetes mellitus and experimental animals with insulin deficiency. Much of the available information regarding the pathogenesis of diabetic nephropathy has been derived from studies using rat models with chemically induced diabetes. The present availability of various rodent models with spontaneous type 2 diabetic renal disease allows investigators to make longitudinal observations on the natural history of the disease in these animals. Rodent models of spontaneous diabetes are also useful for testing the various proposed mechanisms for the development of diabetic glomerulopathy and the effects of various treatment modalities in arresting diabetic nephropathy.

Diversity in manifestations of expression of diabetic renal disease, however, suggests the importance of genetic mechanisms in pathological outcomes. More studies, particularly in genetic animal models of diabetes mellitus, are needed to gain further insight into the role of genetic transmission in diabetic renal disease.

References

1. Seaquist ER, Goetz FC, Rich S, Barbosa J, Familial clustering of diabetic kidney disease. N Engl J Med 320:1162–1165, 1989
2. Mangili R, Bending JJ, Scott G, Li LK, Gupta A, Viberti GC, Increased sodium-lithium countertransport activity in red cells of patients with insulin-dependent diabetes and nephropathy. N Engl J Med 318:146–150, 1988
3. Fabre J, Balant LP, Dayer PG, Fox HM, Vernet AT, The kidney in maturity onset diabetes mellitus: a clinical study of 510 patients. Kidney Int 21:730–738, 1982
4. Humphrey LL, Ballard DJ, Frohnert PP, Chu C-P, O'Fallon M, Palumbo PJ, Chronic renal failure in non-insulin-dependent diabetes mellitus. Ann Intern Med 111:788–796, 1989
5. Velasquez MT, Kimmel PL, Michaelis OE IV, Animal models of spontaneous diabetic kidney disease. FASEB J 4:2850–2859, 1990
6. Hansen BC, Bodkin NL, Schwartz J, Jen KLC, B-cell responses, insulin resistance and the natural history of noninsulin-dependent diabetes in obese rhesus monkeys. In: Shafrir E, Renold AE (eds) Frontiers in diabetes research. Lessons from animal diabetes II. Libbey, London, pp 279–287, 1988
7. Kopp JB, Marinos NJ, Bodkin NL, Hansen BC, Klotman PE, Increased laminin in nodular glomerulosclerosis affecting rhesus monkeys with non-insulin dependent diabetes mellitus (NIDDM). J Am Soc Nephrol 1:551, 1990
8. Cohen AM, Teitelbaum A, Saliternik R, Genetics and diet as factors in the development of diabetes mellitus. Metabolism 21: 235–240, 1972
9. Rosenman E, Cohen AM, The pathology of the Cohen diabetic rat. In: Cohen AM, Rosenmann E (eds) The Cohen diabetic rat. Karger, Basel, pp 54–57, 1990
10. Koletsky S, Obese spontaneous hypertensive rats: a model for study of atherosclerosis. Exp Mol Pathol 19:53–60, 1973
11. Koletsky S, Pathologic findings and laboratory data in a new strain of obese hypertensive rats. Am J Pathol 80:129–142, 1975
12. Ikeda H, Shino A, Matsuo T, Iwatsuka H, Suzuoki Z, A new genetically obese-hyperglycaemic rat (Wistar fatty). Diabetes 30:1045–1050, 1981
13. Kava RA, West DB, Lukasik VA, Greenwood MRC, Sexual dimorphism of hyperglycaemia and glucose tolerance in Wistar fatty rats. Diabetes 338:159–163, 1989
14. Diani AR, Ledbetter SR, Sawada GA, Copeland EJ, Wyse BM, Hannah BA, Peterson T, Structural and functional evidence of diabetic nephropathy in the Wistar fatty diabetic rat. In: Shafrir E, Renold AE (eds) Frontiers in diabetes research. Lessons from animal diabetes II. Libbey, London, pp 535–541, 1988
15. Hansen CT, The development of the SHR/N- and LA/N-*cp* (corpulent) congenic rat strains. In: Hansen CT, Michaelis IV OE (eds) New models of genetically obese rats for studies in diabetes, heart disease, and complications of obesity. Summaries of workshop papers and current bibliography, National Institutes of Health, Bethesda, Md., pp 7–10, 1988
16. Michaelis IV OE, Patrick DH, Hansen CT, Canary JJ, Werner RM, Carswell N, Spontaneous hypertensive/NIH-corpulent rat: an animal model for insulin-independent diabetes mellitus (type II). Am J Pathol 123:398–400, 1986
17. Michaelis IV OE, Ellwood KC, Tulp OL, Greenwood MRC, Effects of feeding sucrose or starch diets on parameters of glucose tolerance in the LA/N-corpulent rat. Nutr Res 6:95–99, 1986
18. Michaelis OE IV, Carswell N, Velasquez MT, Kimmel PL, Abraham AA, Canary JJ, Hansen CT, Influence of genetic obesity, dietary carbohydrate and age on parameters of glucose tolerance and kidney and adrenal gland histology in female SHR/N-corpulent rats. Int J Obesity 14:973–985, 1990
19. Velasquez MT, Kimmel PL, Michaelis IV OE, Carswell N, Abraham AA, Bosch JP, Effect of carbohydrate intake on kidney function and structure in SHR/N-*cp* rats: A new model of NIDDM. Diabetes 38:679–685, 1989
20. Michaelis OE IV, Abraham AA, Kimmel PL, Velasquez MT, Triani RJ, Prazma J, Pathophysiological alterations in diabetic spontaneous hypertensive/NIH-corpulent rats. In: Shafrir E, Renold AE (eds) Frontiers in diabetes research. Lessons from animal diabetes III. Smith Gordon, London, pp 378–380, 1991
21. Michaelis OE IV, Ellwood KC, Emberland JJ, Hansen CT, Canary JJ, Effect of dietary carbohydrate on expression of diabetes in the SHR/N-corpulent rat: A new model for obesity-induced diabetes. J Obesity Weight Reg 4:168–178, 1985
22. Greenhouse DD, Michaelis OE IV, McCune SA, The development of corpulent rat strains. In: Shafrir E (ed) Frontiers in diabetes research. Lessons from animal diabetes III. Smith Gordon, London, pp 375–377, 1991
23. McCune SA, Baker PB, Still HF Jr, SHR/Mcc-*cp* rat: model of obesity, non-insulin dependent diabetes and congestive heart failure. ILAR News 32: 23–27, 1990
24. McCune SA, Jenkins JE, Harold FS Jr, Park S, Radin MJ, Jurin RR, Hamlin RE, Renal and heart function in the SHHF/Mcc-*cp* rat. In: Shafrir E (ed) Frontiers in diabetes research. Lessons from animal diabetes III. Smith Gordon, London, pp 397–401, 1991
25. Michaelis OE IV, Ellwood KC, Judge JM, Hansen CT, Effect of dietary sucrose on the SHR/N-corpulent rat: a new model for insulin-independent diabetes. Am J Clin Nutr 39:612–618, 1984
26. Recant L, Voyles N, Timmers K, Bhathena SJ, Solomon D, Wilkins S, Michaelis OE IV, The effect of the corpulent gene (*cp*) on insulin secretory pattern: comparison of obese non-diabetic LA/N-*cp* and obese diabetic SHR/N-*cp* rats. Diabetes 38: 691–697, 1989
27. Hellman B, Studies in obese-hyperglycaemic mice. Ann NY Acad Sci 131:541–558, 1965
28. Bailey CV, Flatt PR, Radley NS, Effect of high fat and high carbohydrate cafeteria diets on the development of the obese hyperglycaemic (*ob/ob*) syndrome in mice. Nutr Res 5:1003–1010, 1985

29. Like AA, Lavine RL, Poffenbarger PL, Chick WL, Studies in the diabetic mutant mouse. VI. Evolution of glomerular lesions and associated proteinuria. Am J Pathol 66:193–224, 1972
30. Gartner K, Glomerular hyperfiltration during the onset of diabetes mellitus in two strains of diabetic mice (C57BL/6J *db/db* and C57BL/KsJ *db/db*). Diabetologia 15:59–63, 1978
31. Bower G, Brown DM, Steffes MW, Vermier RL, Mauer SM, Studies of the glomerular mesangium and the juxtaglomerular apparatus in the genetically diabetic mouse. Lab Invest 43: 333–341, 1980
32. Nakamura M, Yamada K, Studies on a diabetic (KK) strain of mouse. Diabetologia 3:212–221, 1967
33. Wehner H, Hohn D, Faix-Schade U, Huber H, Walzer P, Glomerular changes in mice with spontaneous hereditary diabetes. Lab Invest 27:331–340, 1972
34. Duhault J, Lebon F, Boulanger M, KK mice as a model of microangiopathic lesions in diabetes. Bibl Anat 11:453–458, 1973
35. Diani AR, Sawada GA, Zhang NY, Wyse BM, Connell CL, Vidmar TJ, Connell MA, The KKAy mouse: a model for the rapid development of glomerular capillary basement membrane thickening. Blood Vessels 24:297–303, 1987
36. Melez KA, Harrison LC, Gilliam JN, Steinberg AD, Diabetes is associated with autoimmunity in the New Zealand obese (NZO) mouse. Diabetes 29:835–840, 1980
37. Renold AE, Spontaneous diabetes and/or obesity in laboratory rodents. In: Levine R, Luft R (eds) Advances in metabolic disorders, vol 3. Academic Press, New York, pp 49–84, 1968
38. Hackel DB, Schmidt-Nielson K, Haines HB, Miai E, Diabetes mellitus in the sand rat (*Psammomys obesus*): pathologic studies. Lab Invest 14:200–207, 1965
39. Marquie G, Duhault J, Jacotot B, Diabetes mellitus in sand rats (*Psammomy obesus*): metabolic pattern during development of the diabetic syndrome. Diabetes 33:438–443, 1984
40. Shafrir E, Teitelbaum A, Cohen AM, Hyperlipidemia and impaired glucose tolerance in *Acomys cahirinus* maintained on synthetic carbohydrate diets. Isr J Med Sci 8:990–992, 1972
41. Orci L, Stauffacher W, Amherdt M, Pictet R, Renold AE, Rouiller CH, The kidney of spiny mice (*Acomys cahirinus*): electron microscopy of glomerular changes associated with aging and tubular glycogen accumulation during hyperglycaemia. Diabetologia 6:342–355, 1970
42. Wise PH, Weir BJ, Hime JM, Forrest E, The diabetic syndrome in the Tuco-Tuco (*Ctenomys talarum*). Diabetologia 8:165–172, 1972
43. Carswell N, Michaelis OE IV, Prather E, Effect of acarbose (Bay-g-5421) on expression of noninsulin-dependent diabetes mellitus in sucrose-fed SHR/N-corpulent rats. J Nutr 119:388–394, 1989

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Review

Molecular mechanism of diabetic nephropathy

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Abstract

Diabetic nephropathy is one of the main causes of renal end-stage disease. Morphologically, the development of diabetic nephropathy is characterized by progressive thickening of the glomerular basement membrane and by expansion of the mesangial matrix which correlates to glomerular filtration function. In vitro studies with cultured mesangial cells revealed that elevated glucose concentrations increase collagen synthesis similar to the in vivo situation. These studies showed that hyperglycemia may be toxic either by non-enzymatic reaction of glucose with proteins and subsequent formation of advanced glucosylation end products or by increased metabolism leading to increased oxidative stress and activation of protein kinase C resulting in increased production of cytokines. Particularly, de novo synthesis of transforming growth factor β1 (TGF-β1) is induced and TGF-β1 appears also involved since blockage of this prosclerotic factor inhibits high glucose-induced collagen synthesis. Interestingly, it could be demonstrated that angiotensin II also stimulates TGF-β1 production possibly via the same signal transduction pathway. Besides the classical clinical chemical parameters for evaluation of renal function, the measurement of urinary albumin excretion is now widely used for detection of developing diabetic nephropathy. Since diabetes causes glomerular and tubular changes, tubular marker proteins may be used to detect early renal damage. An increased urinary excretion of matrix proteins (e.g. collagen) and cytokines (e.g. TGF-β1) was found in early diabetic nephropathy. However, the diagnostic value of these new parameters remains to be established.

Keywords: Diabetic nephropathy; Tubular marker proteins; Matrix proteins; Cytokines; Molecular mechanism

Abbreviations: ACE, angiotensin converting enzyme; AGE, advanced glucosylation end product; RAGE, receptor for advanced glucosylation end product; TGF-β, transforming growth factor-β; PKC, protein kinase C; DCCT, diabetes control and complication trial; UKPDS, United Kingdom prospective diabetes study

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1. Introduction

Diabetes mellitus is now one of the leading causes of renal failure in the Western world. About 40% of all type 1 diabetic patients develop diabetic nephropathy [1]. Since there is a considerable ethnic variability of the incidence of diabetic nephropathy and because not all but only about one third of type 1 diabetics develop diabetic nephropathy a genetic contribution is very likely [2]. Although relatively less type 2 diabetic patients develop nephropathy promoting to end stage renal disease, this group of patients is getting more important because of the rapidly growing number of type 2 diabetic patients. Several large studies particularly the DCCT [3] and the UKPDS study [4] have shown that the progression of diabetic nephropathy may be reduced, but not prevented by strict metabolic control and antihypertensive treatment. Therefore, the knowledge of the molecular mechanism of the pathogenesis of the diabetic nephropathy may help to develop new therapeutic concepts and to find diagnostic markers for early detection of diabetic nephropathy.

2. Structural and functional changes in the course of diabetic nephropathy

After manifestation of diabetes mellitus the first obvious changes are the increase of the kidney and glomerular volume [5,6]. During this early stage the hypertrophic glomeruli show normal structure. Since these changes which are probably caused by hemodynamic factors are potentially reversible they are not a reliable indicator for the development of diabetic nephropathy. Significant structural changes particularly thickening of glomerular basement membrane and mesangial expansion occur only after several years of diabetes. Mogensen and colleagues observed that persistent urinary excretion of minor amounts of albumin are closely linked to the development of diabetic renal disease [7,8]. Electron microscopic studies show that the increase of glomerular extracellular matrix correlates well with the extent of microalbuminuria. This correlation is found in both type 1 and type 2 diabetic patients [9,10]. Detailed immunohistochemical studies have shown that during the development of diabetic glomerulosclerosis there is an increase of the physiologically occurring collagen species [11]. During the development of diabetic nephropathy new types of collagen appear gradually substituting the physiologically occurring collagen types [11–13]. Similar morphologic and structural changes can also be observed in renal interstitium and the glomerular arterioles [14]. Studies in experimental animals have shown that the increase in renal extracellular matrix protein deposition is caused by an increased synthesis of collagens [15]. The increase of glomerular matrix proteins is accompanied by a decrease in basement membrane

associated heparan sulfate proteoglycan [13]. Since this negatively charged proteoglycan represents the anionic barrier of the glomerular basement membrane the decrease of this integral component of the glomerular filtration unit may explain the increased glomerular permeability of albumin. However, this matrix component, which is present in all subendothelial vascular walls [16], is insoluble in physiological fluids and therefore not readily useful for diagnostic purposes. During the cause of diabetic nephropathy the glomerular filtration rate decreases with high individual variability finally leading to end stage renal disease in most type 1 diabetic patients while only 20% of type 2 diabetic patients with diabetic nephropathy progress to end stage disease. Up to now the earliest clinical indication of the development of diabetic nephropathy is the detection of persistent microalbuminuria. Studies of Deckert et al. [1] at the Steno Hospital, Copenhagen, have shown that persistent albuminuria indicates a many-fold increased risk to develop cardiovascular morbidity and mortality. This finding indicates that the presence of persistent microalbuminuria is also an indicator for extrarenal vasculopathies particularly coronary complications.

3. Pathobiochemistry of diabetic nephropathy

Since the development of diabetic nephropathy is faster in patients with bad metabolic control hyperglycemia has been suggested to cause the renal changes [17,18]. Two different mechanisms are currently discussed by which glucose exerts its toxic action:

3.1. Non-enzymatic reaction of glucose

Glucose may react with proteins without enzymatic action by the chemical reactivity of its carbonyl group. This reaction known as non-enzymatic glucosylation or glycation leads to increased addition of glucose to proteins according to the increased glucose levels which is particularly significant in proteins with long life-time, e.g. collagens (Table 1). The glycations of proteins proceeds further resulting in the formation of advanced glycation end products (AGE products) [19]. While the extent of glycation occurring in diabetic patients does apparently not change the biological activity of the proteins the AGE products exert pathophysiological relevant activity [19].

It has been shown that AGEs accumulate in the renal cortex of diabetic rats [20] and in sclerosing glomeruli of diabetic patients [21]. Recent reports indicate that accumulation of AGE in tissues is associated with possibly toxic effects [22]. These include either cross-linking of long-lived matrix proteins or quench nitric oxide, both by chemical reactions. Furthermore, it has been shown that AGEs increase vascular permeability, promote the influx of mononuclear cells,

Table 1
Pathogenetic pathways of glucose toxicity

(1) Non-enzymatic pathways	Glycation of proteins Formation of AGE [19] Induction of oxidative stress via AGE/RAGE interaction [26]
(2) Metabolic pathways of glucose	Sorbitol pathway [18] Hexosamine pathway [30] Increase in oxidative stress [31] Activation of PKC [32,33]

and induce the production of growth factors and cytokines [23]. Search for the cellular and molecular reactions responsible for the AGE-mediated cellular effects has led to the detection of a receptor for AGE (RAGE) [24]. This receptor which has been cloned recently, is expressed on vascular cells which may be involved in the development of diabetic vasculopathies [25]. The cells include macrophages, vascular endothelial and smooth muscle cells and mesangial cells [23,26]. Stimulation of this receptor by AGE-modified proteins results in increased oxidative stress of the cells tested [27]. Furthermore, it has been shown that AGE–RAGE interactions lead to activation of the transcription factor NFκB [28]. Activation of this factor is involved in the expression of genes of the inflammatory response, such as cytokines, growth factor inducable nitric oxide synthesis and RAGE. To reduce the hyperglycemia-induced increased AGE formation in diabetes, inhibitors like aminoguanidine have been developed and appeared beneficial in experimental animals [29]. However, the usefulness of this drug in human diabetic patients remains to be shown.

3.2. Metabolism of glucose

Elevated glucose concentrations may also exert toxic effects by glucose transporter-mediated entrance into the cell and subsequent enzymatic conversion. Several pathways have been identified which are activated upon high ambient glucose concentration (Table 1). These include: activation of the sorbitol pathway, increase in oxidative stress, activation of protein kinase C (PKC), and activation of the hexosamine pathway all of which may lead to increased cytokine and growth factor production. It appears that hyperglycemia-induced increase in renal sorbitol concentration is not a major contributor to the diabetic renal pathology [18,32].

Results of several groups indicate that PKC is activated in vascular cells in diabetes [33,34]. The causal involvement of PKC activation has been further emphasized by treatment of diabetic animals with a new protein kinase C

Table 2
Structure and function of TGF-β1

	Structure	Function
Active TGF-β1	12.5 kDa protein (homodimer)	Prosclerotic (induces synthesis of matrix protein) Immunosuppressive Antiproliferative
Latent TGF-β1	Bound to latency-associated peptide	Biologically inactive

inhibitor (LY-333531, Lilly, Indianapolis, USA) which inhibits the diabetes-induced early (6 weeks) changes in the kidney and in the retina [35].

Increased oxidative stress has also been shown to induce cytokine expression in diabetes. The experiments show that hyperglycemia-induced formation of oxygen radicals is mediated by glucose metabolism. This leads to increased apoptosis as has been recently shown with cultured endothelial cells [31].

The induction of cytokine growth factors have been shown for most of these pathobiochemical pathways in vitro. Therefore the de novo synthesis of several of these factors has been studied in renal tissue of diabetic rats. The results show that transforming growth factor β1 (TGF-β1) is the first and most prominently induced growth factor [36]. The properties of TGF-β1 are shown in Table 2. Recently, the increased glomerular TGF-β1 expression in experimental animals [37] and in human diabetic nephropathy [37,38] has been reported. The involvement of TGF-β1 in the development of early renal changes could be shown by using neutralizing anti-TGF-β1 antibodies [39]. Important to note, Kagami et al. [40] could demonstrate that angiotensin II induces the production of TGF-β1 mRNA, protein and bioactivity in mesangial cells similar to high glucose levels. A possible mechanism is depicted in Fig. 1. This common pathogenetic pathway may explain the beneficial effect of angiotensin converting enzyme (ACE) inhibition in diabetic patients with developing nephropathy.

4. Diagnosis of diabetic nephropathy

Although the natural history of diabetic nephropathy is very complex and not understood in detail, the determination of albuminuria is the most important parameter for the classification of the nephropathy. Clearly microalbuminuria is the earliest parameter for the detection of diabetic nephropathy. Recently, the American Diabetes Association has published a procedure to detect albuminuria in patients [41]. Non-diabetes related renal diseases should be excluded by the clinic using sonography of the kidney. The presence of erythrocytes or

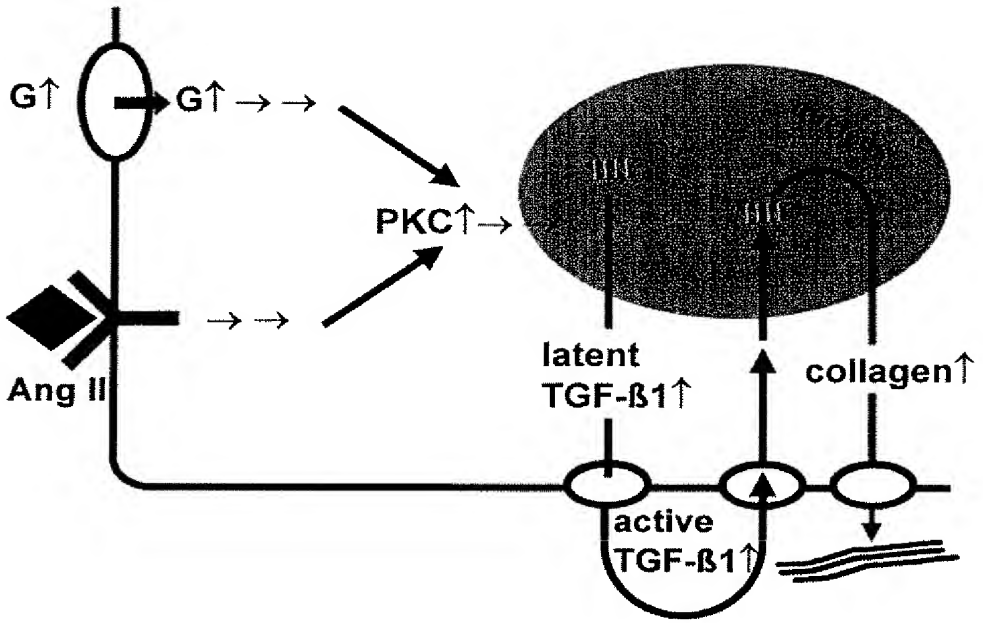

Fig. 1. Schematic drawing of the possible signal transduction pathways activated by hyperglycemia and angiotensin II. Angiotensin II (Ang II) binds to its receptor leading to activation of several signal transduction pathways including activation of protein kinase C (PKC). Similarly high glucose (G) levels lead to activation of PKC although through increased glucose metabolism. The synergistic action of both stimuli may cause de novo synthesis of transforming growth factor-β1 which in turn induces de novo synthesis of, e.g. collagens, leading to the accumulation of extracellular matrix. The spirals in the nucleus indicate the promotor region of the gene which is activated.

leukocytes in urine indicates the presence of renal disease causing albuminuria but may not be related to diabetic nephropathy [42].

5. Clinical value of measurements of new markers

The studies of the pathogenesis of the diabetic nephropathy has led to the investigation of other diagnostic markers for prediction of the development of diabetic nephropathy. Recent studies indicate that urinary excretion of TGF-β1 may be an early indicator for diabetic nephropathy. In the study of Ellis et al. [43] it was shown that urinary TGF-β1 excretion of microalbuminuric patients were elevated by 50% when compared to normal albuminuric patients. Since the individual variation was high, the authors concluded that the urinary excretion of TGF-β1 is not a superior marker compared to albuminuria. In contrast, Japanese

authors found higher TGF-β1 excretion in diabetic patients well correlated to the state of nephropathy [44]. The usefulness of the determination of urinary endothelin excretion is controversely discussed [45]. The current knowledge on blood and urinary markers of diabetic nephropathy has recently been reviewed [46].

Since the epidemiological studies indicate a genetic involvement in the development of diabetic nephropathy several attempts have been made to identify this component. Particularly, ACE polymorphism has been studied as possible genetic marker. However, no significant association of the ACE polymorphism with the development of diabetic nephropathy could be detected [47]. Similarly, the relation of diabetic nephropathy and genetic markers for hypertension, e.g. elevated sodium/lithium and sodium/H^+ transport could be relevant, although this has been also controversially discussed [48].

In conclusion, the current data suggest that glomerular changes seen in long-term diabetes may be caused via increased non-enzymatic reaction of glucose and subsequent formation of AGE or increased metabolism of glucose. Both reactions lead to activation of intracellular signal transduction pathways finally causing increased cytokine production and secretion. Particularly, the hyperglycemia-induced production of TGF-β1 would explain many of the renal changes occurring in developing diabetic nephropathy including matrix accumulation and virtually absent glomerular cell proliferation. Although the recent results suggest new possible markers for the early detection of developing nephropathy up to now the measure of urinary albumin excretion rate is the most reliable indicator.

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References

[1] Borch-Johnson K, Andersen PK, Deckert T. The effect of proteinuria on relative mortality in type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1985;28:590–6.

[2] Pettitt DJ, Knowler WC, Nelson RG. Familial factors in diabetic nephropathy. In: Mogensen CE, editor, The kidney and hypertension in diabetes mellitus, Dordrecht: Kluwer, 1997, pp. 205–14.

[3] The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977–86.

[4] UKPDS 33. An intensive blood glucose control policy with sulphonylureas or insulin reduces the risk of diabetic complications in patients with type 2 diabetes. Lancet 1998;352:837–53.
[5] Mauer SM, Ellis E, Bilous RW, Steffes MW. The pathology of diabetic nephropathy. In: Draznin B, editor, Complications of diabetes mellitus, New York: Alan R. Liss, 1989, pp. 95–101.
[6] Mauer SM, Steffes MW, Ellis EN, Sutherland DN, Brown DM, Goetz FC. Structural–functional relationships in diabetic nephropathy. J Clin Invest 1984;74:1143–55.
[7] Mogensen CE, Christensen CK, Vittinghaus E. The stages in diabetic renal disease, with emphasis on the stage of incipient diabetic nephropathy. Diabetes 1983;32:64–78.
[8] Mogensen CE. Microalbuminuria as a predictor of clinical diabetic nephropathy. Kidney Int 1987;31:673–89.
[9] Osterby R. Glomerular structural changes in type 1 (insulin-dependent) diabetes mellitus: causes, consequences, and prevention. Diabetologia 1992;35:803–19.
[10] Osterby R, Gall MA, Schmitz A, Nielsen FS, Nyberg G, Parving H-H. Glomerular structure and function in proteinuric type 2 (non-insulin-dependent) diabetic patients. Diabetologia 1993;36:1064–70.
[11] Nerlich A, Schleicher ED, Wiest I, Specks U, Timpl R. Immunohistochemical localization of collagen VI in diabetic glomeruli. Kidney Int 1994;45:1648–56.
[12] Kim Y, Kleppel M, Butkowski R, Mauer M, Wieslander J, Michael A. Differential expression of basement membrane collagen chains in diabetic nephropathy. Am J Pathol 1991;138:413–20.
[13] Nerlich A, Schleicher E. Immunohistochemical localization of extracellular matrix components in human diabetic glomerular lesions. Am J Pathol 1991;139:889–99.
[14] Bohle A, Wermann N, Bogenschütz B, Müller CA. The pathogenesis of chronic renal failure in diabetic nephropathy. Pathol Res Pract 1991;187:251–9.
[15] Ceol M, Nerlich A, Baggio B et al. Increased glomerular α1 (IV) collagen expression and deposition in long-term diabetic rats in prevented by chronic glycosaminoglycan treatment. Lab Invest 1996;74:484–95.
[16] Schleicher E, Wagner EM, Olgemöller B, Nerlich A, Gerbitz KD. Characterization and localization of basement membrane associated heparan sulfate proteoglycan in human tissues. Lab Invest 1989;61:323–32.
[17] Ayo SH, Radnik RA, Glass WF et al. Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high-glucose medium. Am J Physiol 1990;260:F185–191.
[18] Schleicher E, Nerlich A. The role of hyperglycemia in the development of diabetic complications. Horm Metab Res 1996;28:367–73.
[19] Brownlee M, Cerami A, Vlassara H. Advanced glucosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 1988;318:1315–21.
[20] Mitsuhashi T, Nakayama H, Ithoh T et al. Immunohistochemical detection of advanced glycation end products in renal cortex from STZ-induced diabetic rat. Diabetes 1993;42:826–32.
[21] Witztum JL, Miyata T, Maeda K et al. Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. Implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. J Clin Invest 1997;100:2995–3004.
[22] Schmidt AM, Yan SD, Stern DM. The dark side of glucose. Nat Med 1995;1:1002–4.
[23] Doi T, Vlassara H, Kirstein M, Yamada Y, Striker GE, Striker LJ. Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. Procl Natl Acad Sci USA 1992;89:2873–7.

[24] Vlassara H, Brownlee M, Cerami A. Novel macrophage receptor for glucose-modified proteins is distinct from previously described scavenger receptors. J Exp Med 1986;164:1301–9.
[25] Schmidt AM, Yan SD, Wautier J-L, Stern D. Activation of receptors for advanced glycation end products. A mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. Circ Res 1999;84:489–97.
[26] Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP. AGEs and their interaction with AGE receptors in vascular disease and diabetes mellitus. Part I. The AGE concept. Cardiovasc Res 1998;37:586–600.
[27] Yan S, Schmidt AM, Anderson GM et al. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. J Biol Chem 1994;269:9889–97.
[28] Bierhaus A, Chevion A, Chevion M et al. Advanced glycation endproduct (AGEs) induced activation of NFκB is suppressed by α-lipoic acid in cultured endothelial cells. Diabetes 1997;46:1481–90.
[29] Soulis T, Cooper ME, Sastra S et al. Relative contributions of advanced glycation and nitric oxide synthase inhibition to aminoguanidine-mediated renoprotection in diabetic rats. Diabetologia 1997;40:1141–51.
[30] Kolm-Litty V, Sauer U, Nerlich A, Lehmann R, Schleicher ED. High glucose-induced transforming growth factor β1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells. J Clin Invest 1998;101:160–9.
[31] Du XL, Sui GZ, Stockklauser-Färber K et al. Induction of apoptosis by high proinsulin and glucose in cultured human umbilical vein endothelial cells mediated by reactive oxygen species. Diabetologia 1998;41:249–56.
[32] Schleicher ED. Biochemical aspects of diabetic nephropathy. In: Mogensen CE, editor, The kidney in hypertension and diabetes mellitus, Dordrecht: Kluwer, 1999, pp. 269–79.
[33] Ayo SH, Radnik R, Garoni JA, Troyer DA, Kreisberg JA. High glucose increases diaclyglycerol mass and activates protein kinase C in mesangial cells. Am J Physiol 1991;261:F571–577.
[34] Larkins RG, Dunlop ME. The link between hyperglycaemia and diabetic nephropathy. Diabetologia 1992;35:499–504.
[35] Ishii H, Jirousek MR, Koya D et al. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. Science 1996;272:728–31.
[36] Nakamura T, Fukui M, Ebihara I et al. mRNA expression of growth factors in glomeruli from diabetic rats. Diabetes 1993;42:450–6.
[37] Yamamoto T, Nakamura T, Noble NA, Ruoslathi E, Border WA. Expression of transforming growth factor β is elevated in human and experimental diabetic nephropathy. Proc Natl Acad Sci USA 1993;90:1814–8.
[38] Iwano M, Kubo A, Nishino T et al. Quantification of glomerular TGF-β 1 mRNA in patients with diabetes mellitus. Kidney Int 1996;49:1120–6.
[39] Sharma K, Ziyadeh FN. Perspectives in diabetes. Hyperglycaemia and diabetic kidney disease. The case for transforming growth factor-β as a key mediator. Diabetes 1995;44:1139–46.
[40] Kagami S, Border WA, Miller DE, Noble NA. Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. J Clin Invest 1994;93:2431–7.
[41] American Diabetes Association. Diabetic nephropathy. Diabetes Care 1998;21:S50–53.
[42] Hofmann W, Edel H, Guder WG. Urineiweiß-Differenzierung. Ein neues Konzept zur nichtinvasiven Diagnostik von Nierenerkrankungen. Münch med Wschr 1997;139:488–94.

[43] Ellis D, Forrest KY, Erbey J, Orchard TJ. Urinary measurement of transforming growth factor-β and type IV collagen as new markers of renal injury: application in diabetic nephropathy. Clin Chem 1998;44:950–6.
[44] Sato H, Iwano M, Akai Y et al. Increased excretion of urinary transforming growth factor beta 1 in patients with diabetic nephropathy. Am J Nephrol 1998;18:490–4.
[45] Mattyus I, Zimmerhackl LB, Schwarz A, Brandis M, Miltenyi P, Tulassay T. Renal excretion of endothelin in children. Pediatr Nephrol 1997;11:513–21.
[46] Hong CY, Chia KS. Markers of diabetic nephropathy. J Diabet Complic 1998;12:43–60.
[47] Pfohl M, Frost D, Koch M et al. Lack of association between the insertion/deletion polymorphism of the angiotensin-converting enzyme gene and diabetic nephropathy in IDDM patients. Horm Metab Res 1998;30:276–80.
[48] Mangili R. Microalbuminuria in diabetes. Clin Chem Lab Med 1998;36:941–6.

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Perspectives in Diabetes

Mesangial Expansion as a Central Mechanism for Loss of Kidney Function in Diabetic Patients

MICHAEL W. STEFFES, RUTH ØSTERBY, BLANCHE CHAVERS, AND S. MICHAEL MAUER

Diabetic nephropathy leading to kidney failure is a major complication of both type I (insulin-dependent) and type II (non-insulin-dependent) diabetes mellitus, and glomerular structural lesions (especially expansion of the mesangium) may constitute the principal cause of decline in kidney function experienced by a significant fraction of diabetic patients. Although the biochemical bases of these mesangial abnormalities remain unknown, an understanding of the natural history of diabetic nephropathy from a combined structural and functional approach can lead to greater pathophysiological insight. Work in animals has supported the concept that the metabolic disturbances of diabetes mellitus cause diabetic nephropathy, with structural and functional lesions prevented or reversed with improved or normalized glycemic control. Additional research must address this fundamental issue in humans, especially the response of advancing mesangial lesions to improved glycemic control. Factors not directly related to the metabolic perturbations of diabetes may serve to accelerate or diminish the pathophysiological processes of diabetic nephropathy. The elucidation and management of these factors, when coupled with improved glycemic control, may moderate the development or progression of diabetic kidney lesions in humans. *Diabetes* 38:1077–81, 1989

Our central postulate is that structural lesions of the glomerulus are the primary cause of the loss of glomerular function and kidney failure in patients with diabetes mellitus. The dominant structural change in patients with advanced diabetic nephropathy is the expansion of the mesangium, including both its cellular and matrix (or basement membrane–like) components (1–3). Enlargement of the mesangium, if not accommodated by an overall growth of the glomerulus, occurs at the expense of the glomerular capillary lumenal space and filtration surface (4,5). This scenario unfortunately happens in a significant fraction (30–40%) of patients with type I (insulin-dependent) diabetes mellitus, who then progress to kidney failure, and often, death (6). Overt diabetic nephropathy with reduced and falling glomerular filtration rate (GFR), proteinuria, and hypertension occurs only in patients with marked mesangial expansion (2) coupled with a significant reduction in glomerular filtration surface (4,5).

The scope of the morphological lesions and accompanying functional abnormalities that we have studied at Århus University and the University of Minnesota spans the natural history of diabetic kidney disease from inception of type I diabetes mellitus (1) through the demise of kidney function (2) to the recurrence of disease in transplanted kidneys (7). Surprisingly, few morphological data are available regarding diabetic nephropathy in patients with type II (non-insulin-dependent) diabetes mellitus (8), but the natural history of diabetic nephropathy may be quite similar in these patients (6,9). A substantial fraction (30–40%) of type I diabetic patients experience diabetic nephropathy leading to kidney failure, but 60–70% do not. This difference may in part be based on differences in glycemic control (10). Long-term, retrospective studies of type I and type II patients indicate that glycemic control is an important although imprecise predictor of diabetic nephropathy risk (9,11,12). Consequently, there is great need to identify other factors in addition to glycemic control that could affect the progression of diabetic nephropathy.

KIDNEY AT EARLY STAGES OF DIABETES MELLITUS

The first major structural change after onset of type I diabetes is enlargement of the whole kidney (13) and individual glo-

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meruli (14). These hypertrophied glomeruli have normal structural composition. The initial characteristic pathological lesions of diabetic glomerulopathy appear only after a few years of diabetes in native kidneys (1,3) and kidneys transplanted to diabetic hosts who had lost kidney function due to end-stage diabetic nephropathy (7). The first detectable structural finding of diabetic glomerulopathy is a widening of the peripheral glomerular basement membrane (GBM), followed by an increase in the fractional volume of the mesangium, i.e., the mesangial volume as a fraction of the total glomerular tuft volume. Functionally, the GFR may be elevated over the first one or two decades, and slightly increased amounts of albumin may be excreted intermittently in the urine (15). Although these early kidney functional manifestations of diabetes mellitus may respond to improvement of glycemic control, the structural lesions in many patients progress over two or more decades of diabetes (16).

INCREASED URINARY ALBUMIN EXCRETION (UAE) AS PREDICTOR FOR DEVELOPMENT OF ADVANCED DIABETIC NEPHROPATHY

Several groups have reported the predictive value of slightly elevated albuminuria (called microalbuminuria) occurring in the first or second decade of type I diabetes as a harbinger of later development of clinical diabetic nephropathy (i.e., albuminuria >300 mg/24 h or ~200 μg/min, hypertension, and falling GFR) (17–20). For the most part, these investigators have used different albuminuria thresholds (from 15 μg/min in ref. 19 to 70 μg/min in ref. 20) to classify the patients retrospectively. The lower value of 15 μg/min lies at the upper limit of normal in most populations, whereas the higher value should increase the specificity by decreasing the number of false-positive samples.

A closer review of these studies (17–20) uncovered microalbuminuria and either rising blood pressure or falling GFR as important concomitants underlying the predictive value of microalbuminuria (21). For example, Mathiesen et al. (20) clearly delineated a susceptible population with progressing albuminuria and rising blood pressure. Thus, it might be argued that microalbuminuria best becomes a predictor of advanced diabetic nephropathy when it coexists with rising blood pressure and/or falling GFR. Microalbuminuria may not be a predictor of nephropathy but in fact may be an early indicator that diabetic nephropathy is already present.

STUDIES OF KIDNEY STRUCTURE AND FUNCTION IN NORMOALBUMINURIC AND MICROALBUMINURIC PATIENTS

We recently explored the relationship of UAE ranging from normal to 200 mg/24 h and morphometric analysis of kidney biopsies to explore the interrelationships of structural and functional kidney changes relatively early in the course of diabetic nephropathy (22). These patients spanned the functional measures seen in the earlier studies of the predictive value of microalbuminuria (17–20): GFR from elevated to normal to subnormal levels, UAE from normal to clearly elevated but undetectable by standard "dipstick" methodology, and/or normal to elevated (and even hypertensive) blood pressure measurements. Patients with normoalbuminuria or microalbuminuria and normal values for creatinine clearance and blood pressure had GBM widths and volume fractions of the mesangium ranging from normal to significantly increased. Thus, normal measures of kidney function in diabetic patients may not indicate normal structure in the kidney. Furthermore, from our previous work in Århus (5) and Minneapolis (2), we would expect clear functional evidence of diabetic nephropathy to appear in some of the patients if the already substantial mesangial expansion progresses. In contrast, microalbuminuria may be present in patients with no measurable kidney structural abnormalities despite >20 yr of diabetes. We would expect such patients to have a good prognosis.

Patients with microalbuminuria and decreased creatinine clearance and/or hypertension uniformly demonstrated widening of the GBM and expansion of the mesangium. In the earlier studies (19,20), many patients with microalbuminuria who proceeded to clinical diabetic nephropathy would have been included in this group on the basis of functional criteria. Thus, microalbuminuria accompanied by hypertension and/or decreased GFR indicates established glomerular structural changes and in this way marks patients who have early clinical nephropathy. Note that such patients in our study regularly had UAE rates >45 mg/24 h (~30 μg/min), and this observation likely explains some of the previously published results (17,18,20). However, it is not clear why one group found a substantially lower UAE rate of 15 μg/min to be a risk discriminator (19).

GLOMERULAR STRUCTURE AND DEMISE OF KIDNEY FUNCTION

We have shown that the area of the peripheral capillary surface is precisely and directly related to the GFR over the spectrum from early to advanced diabetic nephropathy (4,5,14). A typical biopsy in a patient with advanced diabetic nephropathy and declining kidney function will contain hyalinized, obliterated glomeruli and open glomeruli with marked mesangial expansion (Fig. 1). The peripheral capillary surface available for filtration in the open glomeruli and the percentage of nonfunctional, hyalinized glomeruli can be measured. Because the absolute number of glomeruli in a patient cannot be determined, inferences must be made based on the percentage of hyalinized glomeruli. Nevertheless, an ensuing estimation of total peripheral capillary surface per patient (assuming an initially identical number of glomeruli per patient) related well to GFR (4,5,23).

Also, consider the implications of mesangial expansion leading to overt nephropathy against the striking background of the article by Borch-Johnsen et al. (24), who found increased mortality in patients experiencing overt proteinuria. Thus, virtually all of the increased mortality in type I diabetes occurs in patients who develop proteinuria and presumably mesangial expansion, whereas type I diabetic patients without proteinuria have age- and sex-connected mortality rates nearly equivalent to the background nondiabetic population. The increased mortality of the nephropathic type I diabetic patient largely arises from the consequences of uremia and from accelerated macrovascular disease.

Our work with the structural and functional natural history of diabetic nephropathy has identified the expansion of the mesangium and the reduction in peripheral capillary surface as constituting the mechanism leading to the demise in kidney function (2,4,22). GBM width did not correlate with peripheral capillary surface (2). As was true in rats (25), the

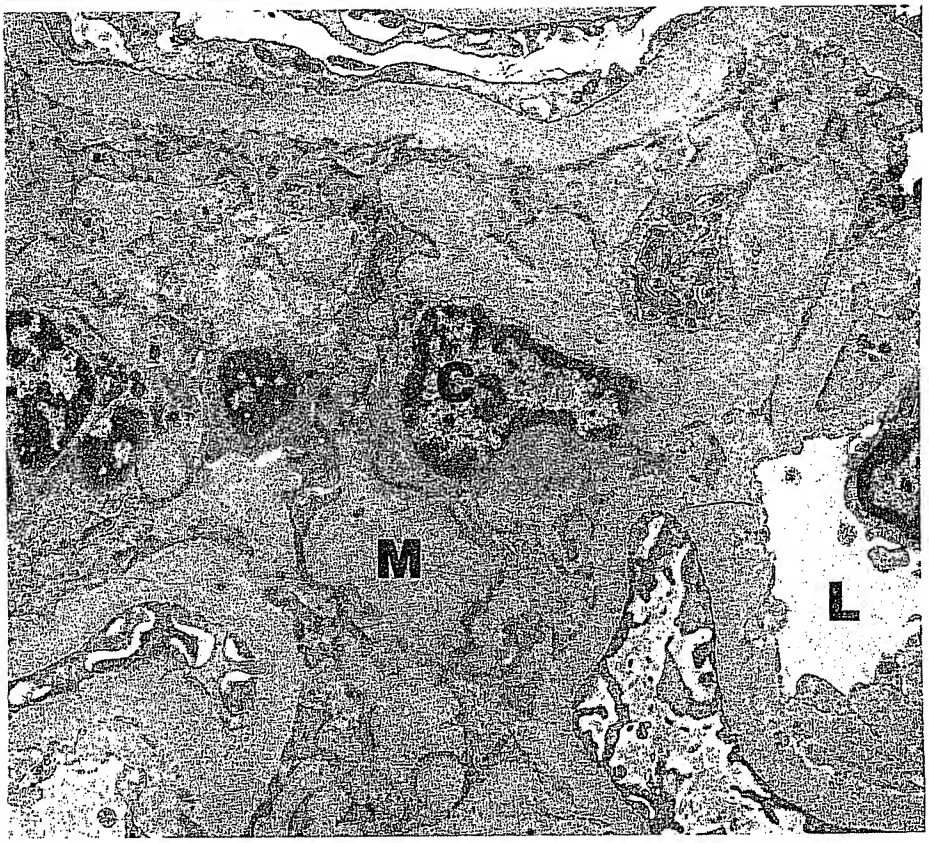

FIG. 1. Electron micrograph of glomerulus from 27-yr-old man diabetic for 15 yr. Note marked amount of mesangium with expansion of both cellular (C) and matrix (*M*) components. *L*, capillary lumen. ×3600.

width of the GBM in diabetic patients does not correlate well with the magnitude of UAE or other measures of kidney function. Normal UAE can occur with a widened GBM in both diabetic patients receiving standard insulin therapy (2) and rodents after successful islet transplantation (25). Diabetic patients experiencing significant proteinuria may not uniformly evince the widest GBMs. Therefore, hypotheses concerning the nature of the matrix constituents of the GBM as the central modulator of the filtration barrier must accommodate the fact that a very thick GBM can be part of a functionally effective barrier. On the other hand, marked mesangial expansion is uniformly associated with proteinuria (2). Yet, like the GBM, the mesangium can undergo expansion beyond the normal range and still not affect kidney function (22); in other words, the functional resiliency of the nephron in the diabetic subject can maintain normal values in standard kidney function tests even with clear structural lesions. One intriguing area to be explored lies at the possible interaction of a markedly expanded mesangium on the barrier integrity of the GBM (26). Little or no information concerns the manner in which the expanded mesangium may change the level of UAE; e.g., why do some patients with demonstrable mesangial expansion still enjoy normal UAE versus others with dipstick-positive proteinuria and similar mesangial lesions?

The role of the mesangium in affecting peripheral capillary surface is best understood when factored by the glomerular volume as Bilous et al. have shown (p. 1142, this issue). It appears that the mesangium expanding into an inherently large or perhaps secondarily enlarged glomerulus will less affect peripheral capillary surface than the same mesangium volume in a smaller glomerulus (4,5,27). This postulate presumes a limited volume within the glomerulus for the structures contained therein. Thus, the change in one component of the glomerulus must affect the volume available to other structures, e.g., glomerular capillaries. Mechanistically the mesangium impinges on the peripheral capillary surface, and with sufficient pathological expansion, the enlarging mesangium will eventually compromise filtration surface and reduce GFR. These structural factors may reflect separate pathophysiological or protective mechanisms affecting the development of diabetic glomerular disease. As an example, the mesangium in a diabetic patient may expand at a rate influenced by the degree of hyperglycemia and the intrinsic response of the mesangium to hyperglycemia. In some patients, at a given level of hyperglycemia, the mesangium may expand very slowly or not at all. In other diabetic patients with similar glycemic control, the mesangium will expand so rapidly as to compromise kidney function. Thus, it appears that the point at which the rapidly enlarging mesangium reduces peripheral capillary surface and thereby compromises kidney function may be determined by the original size of the glomerulus or the capacity of the glomerulus to enlarge when filtration surface is threatened by an enlarging mesangium.

GLYCEMIC CONTROL AND STRUCTURAL LESIONS OF DIABETIC NEPHROPATHY

Whether glycemic control determines the rates of progression or amelioration of the structural or functional abnormalities of diabetic nephropathy in humans remains a major research question. In rats, mesangial lesions and albumi-

nuria can be prevented with optimal glycemic control by insulin injections (28,29; see ref. 25 for review). They can also be reduced to normal levels after successful islet transplantation (30,31). In contrast, GBM widening can be prevented (32) but not reversed (33) with normalization of plasma glucose levels.

Little information has been gathered on the efficacy of improved glycemic control on the progression of the structural lesions of diabetic nephropathy in humans. Retrospective analysis of biopsies from long-term kidney transplants in diabetic patients revealed a threshold of ~13 mM (230 mg/dl) glucose in plasma, above which half but not all kidneys had mesangial expansion by light microscopy (10). Below these very high levels of plasma glucose, measures of glycemia were not correlated to the rate at which the lesions developed. These observations took place against a spectrum of lesions in kidneys transplanted to diabetic hosts that reflected the full range of structural lesions in native kidneys of diabetic patients (2,5,7). Prospective studies to evaluate the efficacy of improved glycemic control on diabetic lesions in native or transplanted kidneys must follow these initial observations.

In addition to the basic issue of glycemic control and the progression or reversal of diabetic glomerular lesions, the magnitude and characteristics of structural change that still can be reversed must be addressed. What stage(s) of mesangial expansion can be diminished or ameliorated with establishment of normoglycemia? The observations of Feldt-Rasmussen et al. (34) may permit an inferential and speculative answer. They demonstrated a halt in the progression of albuminuria with improved but not perfect glycemic control. The level of albumin excretion in their patients, compared to the observations of Chavers et al. (22), suggests the presence of well-established mesangial lesions in the Danish patients. Consequently, the stabilization of albuminuria demonstrated by Feldt-Rasmussen et al. may be compatible with arrest of mesangial lesions, whereas patients with higher levels of glycosylated hemoglobin progressed to overt nephropathy.

FUTURE DIRECTIONS

To better understand the pathophysiological foundation of diabetic nephropathy, we must prospectively observe patients with differing rates of development of disease. Data from animal models of diabetic nephropathy or strictly biochemical studies must be related to the natural history of important structural and functional alterations of the kidney in diabetic patients. Elucidation of the natural history of diabetic nephropathy, manifested by an evolution of biochemical, structural, and functional lesions, remains a primary goal in understanding pathophysiological principles and constructing recommendations for the management of the patient. Given the average two-decade duration over which glomerular lesions destroy kidney function in humans, the goal outlined above is difficult to achieve but necessary to pursue. Although it may be difficult to prove or disprove a hypothesis in patient populations, the application of postulated pathophysiological mechanisms based on biochemical or animal experiments will ultimately depend on research in patients with diabetes. Much of this research will require integrated studies of function and structure revealed by kidney biopsy.

REFERENCES

1. Østerby R: Early phases in the development of diabetic glomerulopathy: a quantitative electron microscopic study. *Acta Med Scand* 574:1–85, 1975
2. Mauer SM, Steffes MW, Sutherland DER, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 74:1143–55, 1984
3. Østerby R: A quantitative electron microscope study of mesangial regions in glomeruli from patients with short-term juvenile diabetes mellitus. *Lab Invest* 29:99–110, 1973
4. Ellis EN, Steffes MW, Goetz FC, Sutherland DER, Mauer SM: Glomerular filtration surface in type 1 diabetes mellitus. *Kidney Int* 29:889–94, 1986
5. Østerby R, Parving H-H, Nyberg G, Hommel E, Jørgensen HE, Løkkegaard H, Svalander C: A strong correlation between glomerular filtration rate and filtration surface in diabetic nephropathy. *Diabetologia* 31:265–70, 1988
6. Andersen AR, Christiansen JS, Andersen JK, Kreiner S, Deckert T: Diabetic nephropathy in type 1 (insulin-dependent) diabetes: an epidemiological study. *Diabetologia* 25:496–501, 1983
7. Mauer SM, Steffes MW, Connett J, Najarian JS, Sutherland DER, Barbosa J: The development of lesions in the glomerular basement membrane and mesangium after transplantation of normal kidneys to diabetic patients. *Diabetes* 32:948–52, 1983
8. Schmitz A, Gundersen HJG, Østerby R: Glomerular morphology by light microscopy in non-insulin-dependent diabetes mellitus: lack of glomerular hypertrophy. *Diabetes* 37:38–43, 1988
9. Nelson RG, Pettitt DJ, Carraher MJ, Baird HR, Knowler WC: Effect of proteinuria on mortality in NIDDM. *Diabetes* 37:1499–504, 1988
10. Mauer SM, Goetz FC, McHugh LE, Sutherland DER, Barbosa J, Najarian JS, Steffes MW: Long-term study of normal kidneys transplanted into patients with type I diabetes. *Diabetes* 38:516–23, 1989
11. Krowlewski AS, Warram JH, Christlieb AR, Busick EJ, Kahn CR: The changing natural history of nephropathy in type I diabetes. *Am J Med* 78:785–94, 1985
12. Deckert T, Poulsen JE, Larsen M: Prognosis of diabetics with diabetes onset before the age of thirtyone: factors influencing the prognosis. *Diabetologia* 14:371–77, 1978
13. Christiansen JS, Gammelgaard J, Frandsen M, Parving H-H: Increased kidney size, glomerular filtration rate and renal plasma flow in short-term insulin-dependent diabetics. *Diabetologia* 20:451–56, 1981
14. Østerby R, Gundersen HJG: Glomerular size and structure in diabetes mellitus: early abnormalities. *Diabetologia* 11:225–29, 1975
15. Mogensen CE: Renal function changes in diabetes. *Diabetes* 25:872–79, 1976
16. Steffes MW, Sutherland DER, Goetz FC, Rich SS, Mauer SM: Studies of kidney and muscle biopsies in identical twins discordant for type 1 diabetes mellitus. *N Engl J Med* 312:1281–87, 1985
17. Viberti GC, Jarrett RJ, Mahmud U, Hills RD, Argyropoulos A, Keen H: Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. *Lancet* 2:1430–32, 1982
18. Parving H-H, Oxenbøll B, Svendsen PAa, Christiansen JS, Andersen AR: Early detection of patients at risk of developing diabetic nephropathy: a longitudinal study of urinary albumin excretion. *Acta Endocrinol* 100:550–55, 1982
19. Mogensen CE, Christensen CK: Predicting diabetic nephropathy in insulin-dependent patients. *N Engl J Med* 311:89–93, 1984
20. Mathiesen ER, Oxenbøll B, Johansen K, Svendsen PAa, Deckert T: Incipient nephropathy in type 1 (insulin-dependent) diabetes. *Diabetologia* 26:406–10, 1984
21. Steffes MW, Chavers BM, Bilous RW, Mauer SM: The predictive value of microalbuminuria. *Am J Kidney Dis* 13:25–28, 1989
22. Chavers BM, Bilous RW, Ellis EN, Steffes MW, Mauer SM: Urinary albumin excretion as a predictor of renal structure in type 1 diabetic patients without overt proteinuria. *N Engl J Med* 320:966–70, 1989
23. Hirose K, Tsuchida H, Østerby R, Gundersen JHG: A strong correlation between glomerular filtration rate and filtration surface in diabetic kidney hyperfunction. *Lab Invest* 43:434–37, 1980
24. Borch-Johnsen K, Andersen PK, Deckert T: The effect of proteinuria on relative mortality in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 28:590–596, 1985
25. Steffes MW, Mauer SM: Diabetic glomerulopathy in man and experimental animal models. *Int Rev Exp Pathol* 26:147–75, 1984
26. Nakamura Y, Myers BD: Charge selectivity of proteinuria in diabetic glomerulopathy. *Diabetes* 37:1202–11, 1988
27. Steffes MW, Mauer SM: Diabetic glomerulopathy: a morphological approach to monitoring development, progression, and reversibility. *Diabetic Nephrop* 4:114–17, 1985
28. Rasch R: Prevention of diabetic glomerulopathy in streptozotocin diabetic

rats by insulin treatment: the mesangial regions. *Diabetologia* 17:243–48, 1979
29. Rasch R: Prevention of diabetic glomerulopathy in streptozotocin diabetic rats in insulin treatment: albumin excretion. *Diabetologia* 18:413–16, 1980
30. Mauer SM, Brown DM, Matas AJ, Steffes MW: Effects of pancreatic islet transplantation on the increased urinary albumin excretion rates in intact and uninephrectomized rats with diabetes mellitus. *Diabetes* 27:959–64, 1978
31. Steffes MW, Brown DM, Basgen JM, Mauer SM: Amelioration of mesangial volume and surface alterations following islet transplantation in diabetic rats. *Diabetes* 29:509–15, 1980
32. Rasch R: Prevention of diabetic glomerulopathy in streptozotocin diabetic rats by insulin treatment: glomerular basement membrane thickness. *Diabetologia* 16:319–24, 1979
33. Steffes MW, Brown DM, Basgen JM, Matas AJ, Mauer SM: Glomerular basement membrane thickness following islet transplantation in the rat. *Lab Invest* 41:116–18, 1979
34. Feldt-Rasmussen B, Mathiesen ER, Deckert T: Effect of two years of strict metabolic control on progression of incipient nephropathy in insulin-dependent diabetes. *Lancet* 2:1300–304, 1986